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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/17, 38/21, 38/28, 38/39

A1

(11) International Publication Number: WO 95/27499

(43) International Publication Date: 19 October 1995 (19.10.95)

(21) International Application Number: PCT/US95/04120

(22) International Filing Date: 7 April 1995 (07.04.95)

(30) Priority Data:
225,372 8 April 1994 (08.04.94) US

(81) Designated States: AU, BR, CA, FI, HU, JP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

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(54) Title: TREATMENT OF AUTOIMMUNE DISEASE USING ORAL TOLERIZATION AND/OR TYPE I INTERFERON

(57) Abstract

The invention is directed to methods for treating autoimmune diseases such as multiple sclerosis by orally administering a bystander antigen such as myelin basic protein or proteolipid protein in conjunction with a polypeptide having Type I interferon activity in such a manner as to induce oral tolerance to the bystander antigen resulting in suppression of the autoimmune response.

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TREATMENT OF AUTOIMMUNE DISEASE USING ORAL TOLERIZATION AND/OR TYPE I INTERFERON

FIELD OF THE INVENTION

This invention pertains to an improvement in the treatment of autoimmune diseases, i.e. in the ability to suppress autoimmune reactions by use of oral tolerization. More specifically, the invention is directed to the oral administration of autoantigens or bystander antigens in combination with oral or parenteral administration of a polypeptide having Type I interferon activity for the treatment of autoimmune diseases.

Yet another aspect of the invention pertains to oral use of interferons in the treatment of autoimmune diseases.

BACKGROUND OF THE INVENTION

Autoimmune diseases are characterized by an abnormal immune response directed against normal autologous (self) tissues.

Based on the type of immune response (or immune reaction) involved, autoimmune diseases in mammals can generally be classified in one of two different categories: cell-mediated (i.e., T-cell-mediated) or antibody-mediated disorders. Non-limiting examples of cell-mediated autoimmune diseases include multiple sclerosis (MS), rheumatoid arthritis (RA), autoimmune thyroiditis (AT), the autoimmune stage of diabetes mellitus (juvenile-onset or Type 1 diabetes) and autoimmune uveoretinitis (AUR). Antibody-mediated autoimmune diseases include without limitation myasthenia gravis (MG) and systemic lupus erythematosus (SLE).

Both categories of autoimmune diseases are currently being treated with drugs that suppress immune responses systemically in a non-specific manner, i.e., drugs incapable of selectively suppressing the abnormal immune response. Non-limiting examples of such drugs include methotrexate, cyclophosphamide, Imuran (azathioprine) and cyclosporin A. Steroid compounds such as prednisone and methylprednisolone (also non-specific immunosuppressants) are also employed in many instances. All of these currently employed drugs have limited efficacy against both cell- and antibody-mediated autoimmune diseases. Furthermore, such drugs have significant toxic and other side effects and, more important, eventually induce "global" immunosuppression in the subject being treated. In other words, prolonged treatment with the drugs downregulates the normal protective immune response against pathogens thereby increasing the risk of infection. In addition, patients subjected to prolonged global immunosuppression have an increased risk of developing severe medical complications from the treatment, such as malignancies, kidney failure and diabetes.

In a continuing effort to overcome the drawbacks of conventional treatments for autoimmune disease, the present inventors and their coworkers have devised methods and pharmaceutical formulations useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments are based on the concept of inducing tolerance, orally or by inhalation, using as the tolerizers autoantigens or bystander antigens or disease-suppressive fragments or analogs of autoantigens or bystander antigens. The present inventors and their co-workers have also devised methods and formulations for inducing tolerance via anergy by the parenteral administration of immunodominant epitopic peptides of autoantigens. This body of work has been described in PCT Application Nos. PCT/US93/01705 filed February 25, 1993, PCT/US91/01466 filed March 4, 1991, PCT/US90/07455 filed December 17, 1990, PCT/US90/03989 filed July 16, 1990, PCT/US91/07475 filed

October 10, 1991, PCT/US93/07786 filed August 17, 1993, PCT/US93/09113 filed September 24, 1993, PCT/US91/08143 filed October 31, 1991, PCT/US91/02218 filed March 29, 1991, PCT/US93/03708 filed April 20, 1993, PCT/US93/03369 filed April 9, 1993, and PCT/US91/07542 filed October 15, 1991.

Autoantigens and bystander antigens are defined below.

Intravenous administration of autoantigens and preferably fragments thereof consisting essentially of immunodominant epitopic regions of their molecules has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy, or T-cell nonresponsiveness, causes deactivation of immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, such as myelin basic protein (MBP), once anergized, no longer proliferate in response to that antigen. The inability of the anergized T-cells to proliferate results in a reduction of the immune attack reactions that cause the tissue damage responsible for the autoimmune disease symptoms, such as the neural tissue damage observed in MS. There is also evidence that oral administration of autoantigens or immunodominant fragments thereof in a single dose and in substantially larger amounts than those that trigger active suppression may also induce tolerance through anergy, or clonal deletion.

Clonal anergy, however, can be induced only when the administered antigen is the specific antigen recognized by the immune attack T-cells sought to be anergized (pure bystander antigens do not induce tolerance through anergy). Thus, regimes that rely on clonal anergy to achieve suppression have certain limitations: the autoantigen may not be known, or there may be several types of immune attack T-cells specific to different antigens, or the antigens to which the immune attack T-cells are specific may change over time.

The present inventors and their co-workers have developed a method of treatment that uses autoantigens and proceeds by active suppression, a different mechanism than

clonal anergy. This method, discussed extensively in the related PCT Application PCT/US93/01705, involves the oral administration of antigens specific to the tissue under autoimmune attack, called "bystander antigens" and defined below. This treatment causes regulatory (suppression) T-cells to be induced in the gut-associated lymphoid tissue (GALT) or, in the case of by-inhalation administration, in the mycosa associated lymphoid tissue (MALT). These regulatory cells are released in the blood or lymphatic circulation and then migrate to the organ or tissue afflicted with the autoimmune disease and suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen recognize at least one antigenic determinant of the bystander antigen used to elicit them and are targeted to the locus of autoimmune attack where they mediate the release of suppressive factors and cytokines, such as transforming growth factor beta (TGF-β) interleukin-4 (IL-4) or interleukin-10 (IL-10). These suppressive substances are indiscriminate and suppress all immune attack phenomena at that vicinity regardless of the antigen that triggers them. (However, because oral tolerization with a bystander antigen causes the release of these nonspecific suppressive substances only in the vicinity of autoimmune attack, no systemic immunosuppression ensues.)

Recently, efforts have also been made by third parties in the treatment of autoimmune disease, specifically MS, involving the parenteral use of the cytokine β-interferon (IFN-β), which is known to have immunomodulatory properties that can be used to advantage in combatting another immune disease. Studies of IFN-β have shown that it tends to inhibit the activity of γ-interferon (IFN-γ). IFN-γ has been shown to exacerbate MS, and may be involved in the pathogenesis of MS lesions. Thus, IFN-β appears to have an effect due in part to its ability to inhibit IFN-γ expression by T-cells. The related ability of IFN-β polypeptide to reduce expression of class II major histocompatibility complex (MHC) molecules on T-cell surfaces, as well as the ability to increase activity of suppressor T-cells, are also thought to be responsible for

the tolerance-promoting immunomodulatory properties of IFN-β. However, parenteral use of IFN-β alone has the disadvantage of requiring high doses, which amplify the known side-effects of this substance.

In Neurology 43:655-661, April 1993, the IFN-β Multiple Sclerosis Study Group reported that subcutaneous administration of either 1.6 X 10^6 or 8 X 10^6 units of IFN-β in patients with relapsing-remitting multiple sclerosis significantly reduced exacerbations of MS compared to a placebo group and, at the higher dose, also decreased MS activity assessed by magnetic resonance imaging. The authors stated that the mechanism by which IFN-β exerted its apparent beneficial effect is not known. IFN-β is neither an autoantigen nor a bystander antigen. Furthermore, the effect of a substance on the immune system of a mammal (as well as the mechanisms by which these effects are brought about) is often different depending on the amount and/or route of administration of that substance. For example, subcutaneous administration of an alloantigen induces an immune response to that antigen. Oral administration of the same substance may induce tolerance by eliciting T-suppressor cells that are specific to the orally administered antigen or (with higher doses and infrequent administration) may induce anergy. Intravenous administration of the same alloantigenic substance may induce tolerance by way of anergy.

To date there has been no teaching of oral use of interferon alone in the treatment of autoimmune disease. Further, there has been no teaching of oral or parenteral use of interferon in conjunction with oral tolerization employing autoantigens or bystanders.

Accordingly, one object of the present invention is to provide an improved and/or more convenient method for treating mammals suffering from autoimmune diseases.

An additional object of the present invention is an improved method for treating mammals suffering from autoimmune diseases exclusively via the oral route.

A third object of the invention is a method for treating mammals suffering from autoimmune diseases through the oral administration of interferons.

SUMMARY OF THE INVENTION

It has now been found that:

- oral or by-inhalation administration of various polypeptides having Type I interferon activity is of benefit in the treatment of autoimmune diseases;
- a combination of (i) oral or by-inhalation administration of autoantigens or bystander antigens (or fragments of them) and (ii) oral or parenteral administration of polypeptides having Type I interferon activity is substantially more effective in the treatment of autoimmune diseases, than administration of (i) or (ii) alone.

Use of other synergists, such as bacterial lipopolysaccharide, Lipid A, choleratoxin β chain etc., as described below, can be conjoined to the foregoing combination.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing the variation in suppression of EAE (induced by MBP peptide 71-90) following oral (1A) or i.v. (1B) administration of different MBP peptides.

Figure 2 (A) is a graph depicting the effect of feeding an autoantigen (PLP) or a bystander antigen (MBP) on EAE induced in SJL/J mice with a PLP-peptide; (B) is a bar graph summarizing the data of (A).

Figure 3 is a bar graph showing the suppression of EAE (induced with MBP-peptide 71-90) by feeding various guinea pig MBP peptides alone or in combination with soybean trypsin inhibitor (STI).

Figure 4 (A-D) are graphic comparisons of the suppression of EAE in Lewis rats with oral administration of guinea pig MBP (A-D), intraperitoneal administration of rat α/β IFN (A), rat mock IFN (C) and combination treatments (B and D).

Figure 5 is a bar graph summary of the data of Figure 4 (A-D).

Figure 6 is a comparative bar graph of an additional experiment documenting the suppression of EAE in Lewis rats with combination of orally administered guinea pig MBP and intraperitoneally administered rat α/β IFN, as well as with MBP or IFN-β alone.

Figure 7 (A-C) are graphic comparisons of the suppression of EAE in SJL/J mice with intraperitoneal administration of mouse IFN-β (A), oral administration of bovine MBP (B), oral administration of bovine PLP (C), and combinations thereof (B and C).

Figure 8 is a bar graph of the suppression of bovine PLP-induced EAE in SJL/J mice with or without intraperitoneal administration of mouse IFN-β; bovine MBP-induced EAE with and without mouse β-interferon; mouse IFN-β alone; and hen egg lysozyme (HEL) (control protein).

Figure 9 is a graphic illustration of the effect of Type II Collagen, or oral IFN-β, or the combination thereof, on the induction of adjuvant arthritis in Lewis rats.

DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of conflict, the description including the definitions and interpretations of the present disclosure will prevail.

Definitions

The following terms, when used in this disclosure, shall have the meanings ascribed to them below:

(a) "Bystander antigen" or "bystander" is a protein, protein fragment, peptide, glycoprotein, or any other immunogenic substance (i.e. a substance capable of eliciting an immune response) that (i) is or is derived from a component specific to an organ or tissue under autoimmune attack; and (ii) upon oral or enteral administration elicits regulatory (suppressor) T-cells (which can be of the CD4+ or CD8+ type) that are targeted to the organ or tissue under attack where they cause at least one immunoregulatory cytokine or immunoregulatory factor (such as IL-4, IL-10, or TGF-β) to be released and thereby suppress immune attack cells that contribute to autoimmune destruction. The destructive cells are suppressed even though they may be specific to a different immunogenic substance from that used to elicit the regulatory cells. The term includes but is not limited to autoantigens (defined below) and fragments thereof involved in autoimmune attack. In addition, the term includes antigens normally not exposed to the immune system, which become exposed in the locus of autoimmune attack as a result of autoimmune destruction of overlying tissue. An example is heatshock proteins, which although nonspecific to a particular tissue are normally shielded form contact with the immune system. "Pure bystander" is a bystander antigen that is not an autoantigen.

(b) "Bystander suppression" is suppression at the locus of autoimmune attack of cells that contribute to autoimmune destruction; this suppression is mediated by the release of one or more immunosuppressive factors from suppressor T-cells elicited by the ingestion or inhalation of a bystander antigen and recruited to the site where cells contributing to autoimmune destruction are found. The result is nonspecific but locally restricted downregulation of the autoimmune responses responsible for tissue destruction.

(c) "Mammal" is defined herein as any organism having an immune system and being susceptible to an autoimmune disease.

(d) "Autoimmune disease" is defined herein as a spontaneous or induced malfunction of the immune system of mammals, including humans, in which the immune system fails to distinguish between foreign immunogenic substances within the mammal and/or autólogous substances and, as a result, treats autologous tissues and substances as if they were foreign and mounts an immune response against them. The term includes human autoimmune diseases and animal models therefor.

(e) "Autoantigen" is any substance or a portion thereof normally found within a mammal that, in an autoimmune disease, becomes the primary (or a primary) target of attack by the immunoregulatory system. The term also includes antigenic substances that induce conditions having the characteristics of an autoimmune disease when administered to mammals. Additionally, the term includes peptide substances consisting essentially of immunodominant epitopes or immunodominant epitope regions of autoantigens. Immunodominant epitopes or regions in induced autoimmune conditions are fragments of an autoantigen that can be used instead of the entire autoantigen to induce the disease. In humans afflicted with an autoimmune disease, immunodominant epitopes or regions are fragments of antigens specific to the organ or tissue under autoimmune attack and recognized by a substantial percentage (e.g. a majority though not necessarily an absolute majority) of autoimmune attack T-cells.

(f) "Treatment" is intended to include both the prophylactic treatment to prevent an autoimmune disease (or to prevent the manifestation of clinical or subclinical, e.g., histological, symptoms thereof), as well as the therapeutic suppression or alleviation of symptoms after the manifestation of such autoimmune disease, by abating autoimmune attack and preventing or slowing down autoimmune tissue destruction.

(g) "Synergists" are defined herein as substances that augment or enhance substantially the suppression of the clinical (and/or subclinical) manifestation of autoimmune diseases when administered in conjunction with the oral administration of a bystander antigen or parenteral administration of an autoantigen. As used in the preceding

sentence, and elsewhere in this specification, "in conjunction with" (or "in association with") means before, substantially simultaneously with, or after oral (or by-inhalation) administration of bystander antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the autoantigen or bystander antigen by so long an interval of time that the relevant effects of the substance administered first have worn off. Therefore, the synergists should preferably be administered within about 24 hours before or after the autoantigen or bystander antigen, and most preferably within about one hour before or after administration of the antigen. Polypeptides having Type I interferon activity are examples of synergists. Examples of other synergists are given below.

(h) "Oral" administration includes oral, enteral or intragastric administration. In addition, by inhalation administration in aerosol form accomplishes the same tolerizing effect and is equivalent to oral tolerization.

(i) "Parenteral" administration includes subcutaneous, intradermal, intramuscular, intravenous, intraperitoneal or intrathecal administration.

(j) "Abatement", "suppression" or "reduction" of autoimmune attack or reaction encompasses partial reduction or amelioration of one or more symptoms of the attack or reaction. A "substantially" increased suppressive effect (or abatement or reduction) of autoimmune reaction means a significant decrease in one or more markers or histological or clinical indicators of autoimmune reaction or disease. Nonlimiting examples are a reduction by at least 1 unit in limb paralysis score or in arthritis score or a significant reduction in the frequency of autoreactive T-cells; a reduction of at least about 0.5 units in insulitis scoring (measured e.g. as described in Zhang et al., PNAS, 1991, 88:10252-10256).

Animal Models

Throughout the present specification, reference is made to various model systems that have been developed for studying autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE) has been studied in mice and other

rodent species as a model for Multiple Sclerosis (MS). Those of ordinary skill in the art recognize that many potential immune therapies for MS are first tested in this animal model system. The disease is induced by immunization with myelin basic protein (MBP) or proteolipid protein (PLP) and an adjuvant (such as Freund's Complete Adjuvant, FCA). The antigen that is used to induce the disease is the autoantigen in the model. This treatment, with either antigen, induces either a monophasic or an exacerbating/remitting form of demyelinating disease (depending on the type and species of rodent and well-known details of induction). The induced disease has many of the characteristics of the autoimmune disease MS and serves as an animal model therefor. Furthermore, the successful treatment of EAE by oral tolerization, and the parallel success in decreasing the frequency of disease-inducing cells in humans, and, in any cases, ameliorating the symptoms of MS, using oral administration of myelin, validates the use of EAE as a model system for predicting the success of different oral tolerization regimens.

Immunization with Mycobacterium tuberculosis or with Freund's Complete Adjuvant in oil into the dorsal root tail of susceptible mammals induces a disease used as a model for human rheumatoid arthritis. In like manner, immunization with Type II collagen with an adjuvant will also induce a disease (collagen-induced arthritis or "CIA") that serves as a model for human rheumatoid arthritis.

Immunization of Lewis rats with S-antigen or IRBP-antigen (InterPhotoReceptor Binding Protein) and an adjuvant induces autoimmune uveoretinitis. Finally, a model for Type I diabetes develops spontaneously in the NOD Mouse and the BB Rat.

One or more of the above disclosed model systems may be employed to demonstrate the efficacy and improved treatment provided by the present invention. In fact, the animal models are particularly suitable for testing therapies involving bystander suppression, precisely because this suppression mechanism is antigen-nonspecific. In the case of oral tolerization, therefore, the suppression of symptoms obtained

in the model is independent of many of the actual or potential differences between a human autoimmune disorder and an animal model therefor. The same animal models are suitable for testing therapies based on use of interferon because interferon is generally known to have the same activities in animal models as in humans.

The above animal models can be thus used to establish the utility of the present invention in mammals (including humans). For example, a multiple sclerosis autoantigen, bovine myelin, orally administered to humans in a double-blind study conferred a considerable benefit to a significant patient subset (Weiner, H. et al. Science 259:1321-1324, 1993). In addition, rheumatoid arthritis symptoms, such as joint tenderness, AM stiffness, grip strength, etc., were successfully suppressed in humans receiving oral collagen (0.1-0.5 mg single dose daily). (Trentham, D. et al., Science 261:1727, 1993.) Finally, preliminary human trials with oral S-antigen showed very encouraging results for uveoretinitis. Large scale human trials are presently conducted for multiple sclerosis, uveoretinitis, rheumatoid arthritis and Type I diabetes. All of these human trials now validate the animal data on oral tolerization using the appropriate disease model. Thus, the predictive value of animal models for oral tolerization treatment of autoimmune diseases has now been substantially supported by these human clinical studies.

What follows is a description of the individual treatments that have now been combined in the treatment method of the present invention. By describing the effect of each of the possible treatments individually, followed by a discussion of the combination treatment, the present specification allows one of ordinary skill to understand the efficacy of these treatments, when combined, to reduce or eliminate tissue damage in autoimmune disease.

Description of Bystander Suppression--Oral Administration

In contrast to clonal anergy, suppression mediated by oral (or by-inhalation) administration of bystander antigens is brought about by elicitation of targetable regulatory T-cells that release one or more nonspecific immunosuppressive

factors, such as transforming growth factor-beta (TGF-β) and/or interleukin 4 (IL-4) and/or interleukin 10 (IL-10) at the locus of the immune attack. These regulatory T-cells do not release IL-2 or γ-IFN. Because regulatory T-cells are elicited, the mechanism at work is known as active suppression. The immunosuppressive substances released by the elicited cells are not specific for the antigen triggering the suppressor cells that release them, even though these regulatory T-cells release immunosuppressive factors only when triggered by the orally administered (or inhaled) antigen. Recruitment of the regulatory T-cells to a locus within a mammal where cells contributing to the autoimmune destruction of an organ or tissue are concentrated allows for the release of immunosuppressive substances in the vicinity of the autoimmune attack and suppresses all types of immune system cells responsible for such attack.

Because the T-suppressor cells have been elicited in response to oral (or by-inhalation) tolerization with a tissue- or organ-specific antigen, the target for the suppressor T-cells is the organ or tissue under immune attack in the particular autoimmune disease where the destructive cells will be concentrated. Thus, the bystander antigen may be an autoantigen or an immunodominant epitope of an autoantigen. Alternatively, the bystander may be another tissue-specific antigen that is not an autoantigen; hence, the autoantigen (or autoantigens) involved need not be identified.

In more detail, the active suppression mechanism of bystander suppression for a tissue-specific (bystander) antigen is as follows: After a tissue-specific bystander antigen is administered orally (or enterally, i.e., directly into the stomach) it passes into the small intestine, where it comes into contact with the so-called Peyer's patches, which are collections of immunocytes located under the intestinal wall. These cells, in turn, are in communication with the immune system, including the spleen and lymph nodes. The result is that suppressor (CD8+ or CD4+) T-cells are induced and recruited to the area of autoimmune attack, where they cause the release of TGF-β and/or another immunoregulatory substances

that downregulate the B-cells as well as the activated helper T-cells directed against the mammal's own tissues. Suppression induced in this manner is antigen-nonspecific. However, the resulting tolerance is specific for the autoimmune disease by virtue of the fact that the bystander antigen is specific for the tissue under attack and suppresses the immune attack cells that are found at or near the tissue being damaged.

Bystander antigens and autoantigens (as well as fragments and analogs of any of them) can be purified from natural sources (the tissue or organ where they normally occur) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual bystander antigens are known: See, e.g., Hunt, C. et al PNAS (USA), 82:6455-6459, 1985 (heat shock protein hsp70); Burkhardt, H., et al., Eur. J. Immunol. 21:49-54, 1991 (antigenic collagen II epitope); Tuohy, V.K., et al., J. Immunol. 142:1523-1527, 1989 (encephalitogenic determinant of mouse PLP in mice); Shinohara, T. et al., In Progress in Retinal Research, Osborne, N. & Chader, J. Eds, Pergamon Press 1989, pp. 51-55 (S-antigen); Donoso, L.A., et al., J. Immunol. 143:79-83, 1989 (IRBP); Borst, D.E., et al., J. Biol. Chem. 264:115-1123, 1989 (IRBP); Yamaki, K. et al., FEBS 234:39-43, 1988 (S-antigen); Donoso, L.A. et al., Eye Res. 7:1087, 1988 (IRBP); Wyborski, R.J., et al., Mol. Brain Res. 8:193-198, 1990 (GAD).

The amino acid sequences for bovine and mouse PLP; bovine, human, chimpanzee, rat, mouse, pig, rabbit, guinea pig MBP; human and bovine collagen alpha-1(II) and bovine collagen alpha-1(I); and human insulin are well-known and published and these antigens can be synthesized by recombinant techniques, as is well-known in the art. Fragments of these antigens can be chemically synthesized or also synthesized by recombinant techniques.

Some tissue-specific antigens are commercially available: e.g. insulin, glucagon, myelin, myelin basic protein, proteolipid protein, collagen I, collagen II, etc.

Bystander antigens can be identified with routine experimentation. Any antigen from the afflicted tissue is a potential bystander. The potential bystander can be fed to mammals, and spleen cells or circulating T-cells from, e.g. the blood or cerebrospinal fluid in the case of EAE or MS, from these mammals can be removed and stimulated in vitro with the same antigen. T-cells elicited by stimulation can be purified and supernatants can be tested for their content of TGF-β, IL-4, IL-10 or other immunoregulatory substances quantitatively and/or qualitatively. In particular, TGF-β can be measured quantitatively or qualitatively, by ELISA using a suitable commercially available polyclonal or preferably monoclonal antibody raised against TGF-β (e.g. one available from RSD Systems, Minneapolis, MN or Celtrix Pharmaceuticals, Santa Clara, CA). Alternatively, another known assay for TGF-β detection can be employed, such as that described in Example 2 below using a commercially available mink lung epithelial cell line. If the bystander antigen elicits T-suppressor cells that do not release TGF-β, the T-cells can be similarly tested for secretion of IL-4 or IL-10. (Antibodies to IL-4 and IL-10 are commercially available, e.g. from Pharmigen, San Diego, CA.) Tissue-specific antigens that are not effective bystander antigens are those so segregated from the inflammatory locus so that the immunoregulatory factors or cytokines released will be too far removed from the locus of inflammation to exert a substantial suppressive effect. Efficacy of interferon or conjoint therapy can be assessed using the same methods.

The efficacy of bystander suppression induced orally or by inhalation can be assessed, e.g., by: diminution in certain inflammation markers, such as the number of activated T-cell clones directed against the organ or tissue that is the target of autoimmune attack; decrease in IL-2 or IFN-γ levels at the same locus; histological evaluation of the afflicted organ or tissue (e.g., by biopsy or magnetic resonance imaging); or reduction in the number and/or severity of clinical symptoms associated with an autoimmune disease.

Use of Bystander Antigens - Dosages

The tolerance induced by the bystander antigens of this invention is dose-dependent over a broad range of oral (or enteral) or inhalable dosages. However, there are minimum and maximum effective dosages. In other words, active suppression of the clinical and histological symptoms of an autoimmune disease occurs within a specific dosage range, which, however, varies from disease to disease, mammal to mammal, and bystander antigen to bystander antigen. For example, when the disease is PLP-induced EAE in mice, the suppressive dosage range when MBP is used as the bystander is from about 0.1 to about 1 mg/mouse/feeding (with feedings occurring about every other day e.g., 5-7 feedings over a 10-14-day period). A most preferred dosage is 0.25 mg/mouse/feeding. For suppression of the same disease in rats, the MBP suppressive dosage range is from about 0.5 to about 2 mg/rat/feeding and the most preferred dosage is 1 mg/rat/feeding. The effective dosage range for humans with MS, when MBP is used as the oral tolerizer, is between about 1 and about 100, preferably between about 1 and about 50 mg MBP per day (administered every day or on alternate days for a period of time ranging from several months to several years) with the optimum being about 30 mg/day.

For rheumatoid arthritis, the effective dosage range for humans receiving either Type I or II collagen is about 0.1 to about 1 mg/day, preferably 0.1-0.5 mg/day. For adjuvant-induced arthritis in mice the effective collagen dosage range is about 3 to about 30 micrograms/feeding with the same feeding schedule as for EAE.

Monitoring of the patient is desirable in order to optimize the dosage and frequency of administration. The exact amount and frequency of administration to a patient may vary depending on the stage, frequency of manifestation and severity of the patient's disease and the physical condition of the patient, as is well-appreciated in the art. Such optimization is preferably effected on a case-by-case basis. Optimization of the dosage necessary for immune suppression involves no more than routine experimentation, given the guidelines disclosed herein.

Assessment of the disease severity can be accomplished according to well-known methods depending on the type of disease. Such methods include without limitation:

MS: severity and number of attacks over a period of time; progressive accumulation of disability (which can be measured e.g. on the Expanded Disability Status Scale); number and extent of lesions in the brain (as revealed, e.g., by magnetic resonance imaging); and frequency of autoreactive T-cells.

EAE: limb paralysis which can be scored as follows: 0-no disease; 1-decreased activity, limp tail; 2-mild paralysis, unsteady gait; 3-moderate paraparesis, limbs splayed apart; 4-tetraplegia; and 5-death.

RA: joint swelling, joint tenderness, morning stiffness, grip strength, joint imaging techniques.

AUR: visual acuity; number of T-cells in the eye and "cloudiness" in the eye.

Type I Diabetes: pancreatic beta cell function (assessed, e.g., by OGTT glucose tolerance test).

NOD Model: insulitis and delay of diabetes onset.

CIA: Arthritis score based on number of affected joints in each of four paws and grading each on an arbitrary scale of 1-4 as follows: 0=normal; 1=redness only; 2=redness plus swelling; 3=severe swelling; and 4=joint deformity. The total arthritis score is the sum of the scores for all paws. Maximum arthritis score is the highest score for an animal over the course of the disease. According to this grading method the highest arthritis score possible is 16 (4 paws X 4 score-per-paw). Stabilization of symptoms, under conditions wherein control patients or animals experience a worsening of symptoms, is

one indicator of efficacy of a suppressive treatment.

Another measure of improvement is the dose reduction or discontinuance of other medications, e.g., steroids or other anti-inflammatory medications, and biologic response modifiers such as methotrexate, subcutaneous interferon and the like.

The optimum dosage of a bystander antigen will be the one generating the maximum beneficial effect assessed as described above.

An effective dosage will be one that causes at least a statistically clinical significant attenuation of at least one marker, symptom or histological evidence characteristic of the disease being treated.

When combined with IFN-β treatment, the dosage of bystander antigen should be equal to that which would have been used if oral or enteral administration of the bystander antigen was used alone, except that the combination is more effective in abating autoimmune reaction, and thus suppressing disease. However, it is possible to decrease the amount of autoantigen or bystander antigen when Type I interferon is conjoined to oral bystander therapy. Furthermore, it is possible to use suboptimal amounts of interferon β in conjoined therapy. A suboptimal amount is an amount of IFN-β which although substantially ineffective when administered alone still enhances the tolerizing ability of the antigen in conjoint therapy, i.e. has a potentiating effect.

Ascertaining the effective dosage range as well as the optimum amount of bystander antigen is well within the skill in the art. For example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose (e.g., 1 microgram), progressively increasing it (e.g. logarithmically) and measuring the number of TGF-beta (and/or IL-4 and/or IL-10) secreting cells and/or assessing the number and activation of immune attack T-cells in the blood (e.g. by limiting dilution analysis and ability to proliferate) and/or assessing the disease severity, as described above. The optimum dosage will be the one generating the maximum amount of suppressive cytokines in the blood and/or causing the

greatest decrease in disease symptoms. An effective dosage range will be one that causes at least a statistically or clinically significant attenuation of at least one symptom characteristic of the disease being treated.

The maximum effective dosage of a bystander can be ascertained by testing progressively higher dosages in animals and then extrapolating to humans. For example, based on the dosages given above, the maximum effective dose of MBP for humans has been estimated at between about 50 and 100 mg. Similarly, the maximum effective Collagen II dose for humans is estimated at about 1 mg/day.

The present invention can also be advantageously used to prevent the onset of an autoimmune disease in susceptible individuals at risk for an autoimmune disease. For example, methods for the identification of patients who are at risk for developing Type 1 diabetes are extant and reliable and have been recently endorsed by the American Diabetes Association (ADA). Various assay systems have been developed which (especially in combination) have a high predictive value assessing susceptibility to Type 1 diabetes (Diabetes Care 13: 762-775, 1990. Details of one preferred screening test are available to those of ordinary skill in the art (Bonifacio, E. et al., The Lancet 335: 147-149, 1990).

From a practical point of view, preventing the onset of most autoimmune diseases is of most importance in the case of diabetes. Other autoimmune diseases MS, RA, AT and AUR are declared at an earlier stage of tissue destruction, before substantial tissue damage has taken place; therefore preventive treatment of these diseases is not as important as in the case of diabetes. In diabetes, it is important to intervene with an effective treatment prior to the substantial destruction of substantially all of the pancreatic islet cells. After the islet cells are destroyed, the treatment would not be effective.

A non-limiting list of autoimmune diseases and tissue- or organ-specific confirmed or potential bystander antigens effective in the treatment of these diseases when administered in an oral or inhalable form are set forth in

Table 1 below. Administration of combinations of antigens listed for each individual disease (with or without conjunction with Type I interferon) is also expected to be effective in treating the disease.

Bystander antigens can be administered by inhalation and so can Type I interferon. The bystander amounts that need to be inhaled are generally smaller than those for oral administration. It is anticipated that the Type I interferon amounts that need to be administered by inhalation will be likewise smaller. Effective amounts can be assessed using the same methodologies provided above.

Table 1

Autoimmune Disease	Affected Tissue	Bystander Antigen	Source	Type
Type 1 Diabetes	pancreatic beta cells	glucagon insulin glutamic acid decarboxylase (GAD)		
Multiple Sclerosis	myelinated neurons	MBP, MBP fragments PLP, PLP fragments myelin-assoc glycoprotein myelin, oligodendrocyte glycoprotein, heatshock protein	J.Chromatog. Biomed.Appl. 526:535 (90)	purification
Rheumatoid Arthritis	connective tissue	RO/SS-A RO/SS-B-LA heatshock protein collagen I, II, III	J.Immunol.Meth 121:219 (89) 151:177 (92)	purification purification
Autoimmune uveitis	eye	S-antigen IRBP recoverin	Exp.Eye Res. 56:463 (93)	cDNA
Myasthenia Gravis	muscle	acetylcholine receptor heatshock protein	Eur.J.Pharm. 172:231 (89)	purification
Male Infertility	sperm	NASP(post-acrosomal sperm protein)	Biol.Reprod. 43:559 (90)	cDNA
Myositis	muscle	Jo-1 antigen	Biol.Chem.H-S. 368:531 (87)	purification

Autoimmune Disease	Affected Tissue	Bystander Antigen	Source	Type
Pemphigus	skin	desmoglein Factor XIII	Eur.J.Cell Biol. 55:200 (91)	cDNA

For any autoimmune disease, extracts of the relevant tissue, as well as specific bystander antigens or fragments thereof, can be used as oral tolerizers. In other words, the bystander need not be purified. For example, myelin has been used for MS, pancreatic cell extracts have been used for Type 1 diabetes, splenic cell extracts have been used to prevent allograft rejection (which is not, strictly speaking, an autoimmune phenomenon) and muscle extracts have been used to treat myositis. However, administration of one or more individual antigens or fragments is preferred.

Thus, according to the present invention, when treating Type 1 diabetes, an effective amount (determined as described above) of glucagon can be administered orally. Glucagon is specifically present in the pancreas. Glucagon, however, is clearly not an autoantigen because it is not expressed in pancreatic beta cells which are destroyed in the course of Type 1 diabetes (glucagon is found exclusively in alpha cells, a different cell type). Thus, glucagon is a "pure" bystander: it does not appear to have any autoantigen activity. (Presumably, the bystander activity of glucagon results from its high local concentration in the pancreatic intercellular milieu due to its secretion from alpha cells.)

Insulin has bystander activity for Type 1 diabetes. It is not at present known whether insulin is also an autoantigen. However, whatever the mechanism of action, oral, enteral or inhalable insulin preparations are effective in suppressing Type 1 diabetes and animal models therefor by preventing autoimmune distinction of pancreatic beta cells.

For multiple sclerosis and its animal models, both disease-inducing and non-inducing fragments of MBP (e.g. a peptide comprising guinea pig MBP amino acids 21-40 which is known not to induce EAE in mice or rats) have bystander activity not only for MBP-induced disease but also for PLP-induced disease. In rats, feeding of bystander generates mostly $CD8^+$ suppressor cells which are Class I restricted whereas in mice both $CD8^+$ and $CD4^+$ regulatory cells are generated (these $CD4^+$ cells are probably Class II restricted).

For rheumatoid arthritis and animal models therefor, Type-I, Type-II and Type-III collagen are known to have bystander activity.

For uveoretinitis and its animal model AUR, S-antigen and IRBP and fragments thereof have bystander activity.

Fragments of bystander antigens can also be employed. Useful fragments can be identified using the overlapping peptide method of Example 3 (which is a general technique although in Example 3 it is described specifically with respect to identification of noninducing fragments of MBP). T-cells from fed animals can be tested for secretion of TGF-β, and/or IL-4 and/or IL-10 and can further be identified by subtype ($CD8^+$ and/or $CD4^+$).

Orally administered autoantigens and bystander antigens elicit regulatory T-cells and thereby induce the production and/or release of TGF-β and/or IL-4 and IL-10. One such T-cell has been identified in mice orally tolerized against EAE as a CD4+ suppressor T-cell, and a CD8+ suppressor T-cell has been identified in rats. Even immunodominant epitopes of autoantigens, e.g. MBP, are capable of inducing such regulatory T-cells. Additional such epitopes can be identified by feeding a bystander antigen to a mammal and isolating from the mammal T-cells that recognize a fragment of the antigen (and thus identifying suppressive fragments), or by identifying T-cells from a bystander fed mammal that can adoptively transfer protection to naive (not-fed) animals.

The bystander antigens can be administered individually or in combinations of at least two. Autoantigen and bystander administration is carried out as disclosed in PCT Applications PCT/US93/01705 filed February 25, 1993, PCT/US91/01466 filed March 4, 1991, PCT/US90/07455 filed December 17, 1990, PCT/US90/03989 filed July 16, 1990, PCT/US91/07475 filed October 10, 1991, PCT/US93/07786 filed August 17, 1993, PCT/US93/09113 filed September 24, 1993, PCT/US91/08143 filed October 31, 1991, PCT/US91/02218 filed March 29, 1991, PCT/US93/03708 filed April 20, 1993, PCT/US93/03369 filed April 9, 1993, and PCT/US91/07542 filed October 15, 1991 mentioned above. It is anticipated that

administration of at least two bystander antigens (either or both of which may but need not be an autoantigen) will also result in effective suppression of autoimmune disease.

In addition, other synergists can be conjoined in the treatment to enhance the effectiveness of the above. Non-limiting examples of noninterferon synergists for use in the present invention include bacterial lipopolysaccharides from a wide variety of gram negative bacteria such as various subtypes of E. coli and Salmonella (LPS, Sigma Chemical Co., St. Louis, MO; Difco, Detroit, MI; BIOMOL Res. Labs., Plymouth, PA), Lipid A (Sigma Chemical Co., St. Louis, MO; ICN Biochemicals, Cleveland, OH; Polysciences, Inc., Warrington, PA) and immunoregulatory lipoproteins, such as peptides covalently linked to tripalmitoyl-S-glycarylcysteinyl-seryl-serine (P_3 C55) which can be obtained as disclosed in Deres, K. et al. (Nature, 342:561-564, 1989) or "Brauns" lipoprotein from E. coli which can be obtained as disclosed in Braun, V., Biochim. Biophys. Acta 435:335-337, 1976. LPS for use in the present invention can be extracted from gram-negative bacteria and purified using the method of Galanes et al. (Eur. J. Biochem. 9:245, 1969) and Skelly, R.R., et al. (Infect. Immun. 23:287, 1979). The effective dosage range for noninterferon synergists for mammals is from about 15 μg to about 15 mg per kg weight and preferably 300 μg - 12 mg per kg weight. Any other substance which has the property of biasing immune responses towards Th2-type response can be used.

Interferons Useful in the Present Invention

Interferons (IFNs) are a diverse family of proteins that are secreted by virtually every cell type within the body in response to a variety of inducers. There are three major species of IFN: α, β, and γ, which are respectively induced from lymphocytes, from fibroblasts (or other nonleukocyte cells), or from lymphocytes that have been stimulated with antigen or mitogen. Interferons are known to affect immunological responses at multiple cellular targets through binding to cellular receptors. IFN α and β are both Type I interferon.

Human IFN-α refers to a group of at least 14 structurally related polypeptides that are transcribed from a multigene family. Human IFN-β refers to two IFN subtypes, which are structurally related to IFN-α, and IFN-β1 is the major subtype. A single receptor binds both IFN-α and IFN-β. (Wierenga et al. "Lymphokines and Cytokines of the Immune System" Ch. 15, Comprehensive Medicinal Chemistry, vol. 3, pp. 1101-1128, 1990). The amino acid sequences of human and rodent α and β interferons have been published. Also published are a number of polypeptides that have Type I interferon activity. See, e.g. U.S. Patent Nos. 4,569,908; 4,793,995; 4,914,033; 4,769,233; 4,753,795; 4,738,845; 4,738,844; 4,914,031 and 4,652,639 (consensus interferon from Amogen, Inc.). A number of human and animal polypeptides having Type I interferon activity are commercially available.

Treatment with Type I Interferon

Clinical trials for parenteral treatment with IFN-β of relapsing and remitting MS, an autoimmune disease, resulted in a significant decrease in attack rate (Jacobs et al., Science 214:1026-1028, 1981). In this trial, 1 million units of IFN-β were administered intrathecally once or twice a week, followed by monthly dosages. More recently, clinical trials with recombinant IFN-β also administered parenterally have also resulted in significantly lower attack rates (Johnson et al., Neurology 40 (Suppl. 1): 261,1990). This trial utilized various initial dosages, but followed the initial dose with subcutaneous administration of 45 million units three times a week. The recombinant IFN-β used in this trial had been altered from the native protein in that the cysteine at position 17 had been changed to serine (IFN-β-ser-17, BETASERON®, Berlex Laboratories). This mutation greatly increases the stability of the protein, yet does not alter the specific activity. A large multicenter trial based on the promising results of Johnson et al. is currently in progress. An additional large trial using a glycolsylated recombinant IFN-β produced in mammalian cells (BIOFERON®) is also ongoing.

Although the mechanism of action of IFN-β is still uncertain, it is probable that IFN-γ is an activator of disease

activity and IFN-β reduces disease symptoms through suppression of its effects. It is known that IFNs as a class can modulate the immune system through induction of MHC class I(HLA-A, B, and C) and class II (HLA-DR, DQ, and DP) cell surface molecules. These surface molecules are essential for basic immune functions such as self/nonself discrimination and antigen presentation to T-cells and thus are thought to play a central role in autoimmune disease development.

Antigens associated with class I molecules are recognized by $CD8^+$ (suppressor/cytotoxic) T-cells, whereas antigens associated with class II molecules are recognized by $CD4^+$ (helper/inducer) T-cells. Class I molecules are constitutively expressed on nearly all cells, and all IFN types augment their expression. Class II molecules are normally restricted to certain cell types, where IFN-γ regulates expression (McFarlin, Allergy Clinics of North America 8:210-212,1988; Basham et al., J. of Immunol. 130:1492-1494,1983; Sztein et al., J. Clin. Invest. 73:556-565,1984). This regulation is thought to occur through induction of intracellular proteins which, in turn, mediate MHC molecule expression (Vanguri et al. J. of Biol. Chem. 265:15409-15057, 1990). IFN-γ also activates marcrophages, which act as effector cells in autoimmune attacks, such as the demyelination seen in MS (Bever et al., Springer Sem. in Immunopathol. 8:235-250,1985; Prineas et al., Ann. of Neurol. 10:149-158, 1981). Macrophages also synthesize proteinases that can degrade autoantigens, such as MBP (Bever, Transact. of the Am. Soc. Neurochem. 25:208,1991). IFN-γ can also induce adhesion molecules which regulate homing of lymphocytes to sites of inflammation as is seen in autoimmune disease, for example, the CNS in MS (Male et al., Cell. Immunol. 1271-11,1990). Thus, this cytokine has been implicated in several aspects of autoimmune disease.

The immune activating effects of IFN-γ are modulated by other cytokines. For example, interleukin-4, corticosteroids, prostaglandins, α-fetoprotein, TGF-β, and noradrenaline have all been shown to downregulate class II expression in various experimental systems (Cowan et al., J.

of Neuroimmunol. 33: 17-28,1991; Frohman et al., Proc. Natl. Acad. Sci. USA 85:1292-1296,1988; Racke et al., J. of Immunol. 146:3012-2017,1991; Ransohoff, Res. in Immunol. 140:202-207,1989). Interestingly, IFN-γ stimulated class II molecules can also be downregulated by IFN-β, which has been shown to interfere with transcription of class II-specific mRNA in several systems (Fertsch et al., J. of Immunol. 139:244-249,1987; Ransohoff et al., J. of Neuroimmunol. 33:103-112,1991). It has been shown in T lymphocytes that IFN-β directly suppresses the synthesis of IFN-γ, which may explain its effect on class II gene transcription (Noronha et al., Neurology 41 (Suppl. 1):219,1991; Pantich et al., Ann. of Neurol. 22:139,1987). As class II molecules are known to be central to the recognition of cells as foreign, they almost certainly play a major role in the pathology of autoimmune disease.

Administration of IFN-γ to MS patients can precipitate acute exacerbations (Pantitch et al., supra). IFN-γ is also present in association with class II antigens in active MS plaques (Traugott et al., N.Y. Acad. Sci. 540:309-311,1988). Further, recombinant IFN-β has been shown to improve suppressor function of T-cells from both MS patients and control subjects, possibly through a regulation of IFN-γ synthesis by the suppressor T-cells (Noronha et al., Ann. of Neurol. 27: 207-210,1990; Panitch et al., J. of Neuroimmunol. 1992). Downregulation of IFN-γ by systemic IFN-β could thus be an effective means of preventing or modulating the severity of autoimmune attacks.

This view has also been supported by work in animal models. Specifically, in EAE, the model for MS discussed above, systemic administration of IFN-β has prevented the development of the disease (Abreu, Immunological Communications 11:1-7, 1982). Adoptive transfer of the disease, that is, inducing the disease through the transfer of MBP-sensitized T-cells from rats suffering from EAE to normal rats, is prevented if the cells are incubated with IFN-β before transfer (Abreu, Internl. Arch. of Allergy and App. Immunol. 76:302-207, 1985). In both cases it is likely that these effects are due to

downregulation of the rat Ia molecule, which corresponds to human class II MHC, although this has not been proven.

Oral Use of Type I IFN Alone in the Present Invention

Type I interferons orally administered are effective in suppressing autoimmune disease. In fact, IFN-β has been as effective as insulin in suppressing diabetes in NOD mice. This fact is surprising and cannot be inferred from parenteral use of IFN-β as the mechanism by which interferon suppresses disease is unknown.

For rats and mice, oral dosages that have been found effective range between 1,000 and 150,000 units with no maximum effective dosage having been discerned. This contrasts with the response to bystander antigens, which declines above a maximum effective dose. It is expected that the dosages employed with IFN alone are similar to those employed in combination with bystander antigens, except that suboptimal dosages of Type I interferon can also be used in conjoint therapy. Unlike parenteral interferon, there are no side effects with oral interferon.

Parenteral IFN Dosages (Combination Treatment)

The reduction of autoimmune disease symptoms seen with the administration of Type I IFN alone is effective over a broad range of parenteral dosages. In other words, suppression of the clinical and histological symptoms of an autoimmune disease occurs within a specific dosage range, which, however, varies from disease to disease, mammal to mammal and the form and activity of IFN. For example, when the disease is PLP- or MBP-induced EAE in mice, the suppressive dosage range when mice IFN-β is used is from about 10,000 to 1 million units (with treatments occurring about every other day (e.g., 5-7 treatments over a 10-14-day period). A most preferred dosage is 69,000 units/mouse/treatment. For suppression of the same disease in rats, the IFN-β suppressive dosage range is from about 5,000 to about 1 million units/rat/treatment and the most preferred dosage is 15,000 units/rat/treatment. The effective dosage range for humans with MS is between about 1 million units and about 75 million units, preferably between about 15 and about 50 million units

per dose, administered as infrequently as monthly and as frequently as every other day.

Ascertaining the effective dosage range for conjoint therapy as well as the optimum amount is well within the skill in the art. For example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose (e.g., 5,000 units), progressively increasing it (e.g. logarithmically) and measuring a biological reaction to the treatment, for example reduction in class II surface markers on circulating T-cells and/or by scoring the disease severity, according to well-known scoring methods (e.g., on a scale of 1 to 5, or by measuring the number of attacks, or by measuring joint swelling, grip strength, stiffness, visual acuity, ability to reduce or discontinue medication, etc. depending on the type of disease). The optimum dosage will be the one having the greatest influence on the biological phenomenon being measured, such as that which causes the greatest reduction in class II molecules on the T-cell surface, and/or that which causes the greatest decrease in disease symptoms. An effective dosage range will be one that causes at least a statistically or clinically significant attenuation of at least one symptom characteristic of the disease being treated, as discussed hereinabove. Again, suboptimal dosages of parenteral interferon can be used in conjoint therapy.

Parenteral administration may be via subcutaneous, intramuscular, or intraperitoneal, routes, with subcutaneous being preferred for treatment purposes if the parenteral route is selected. In the case of parenteral administration, interferon may be formulated in sterile saline or other carriers well known in the art, and may include excipients and stabilizers that are standard in the art.

Combination Therapy

It has been surprisingly discovered that the oral (or by inhalation) administration of a bystander antigen in conjunction with oral or parenteral administration of Type I IFN, results in a treatment which is synergistic in its effect

on autoimmune disease, when compared to the effect of the two treatments separately.

This treatment has been studied both in rats and mice, using the animal model for MS, EAE, the animal model for RA (IA), and the animal model for Type I diabetes, NOD. The experimental protocol for these studies is disclosed in the Examples below. As seen in Figure 4, treatment of Lewis rats with orally administered guinea pig MBP after induction of EAE both delays the onset and reduces the clinical score of the disease (see A-D). The intraperitoneal administration of rat IFN-β alone appears to have almost no effect on the timing or severity of the disease (see 8A). This treatment is directly comparable the effect of administration of mock rat IFN-β (see 8C), a control substance which is produced by subjecting cells to the same growth conditions as those producing IFN-β but in the absence of an inducer.

However, treatment with a combination of intraperitoneal rat IFN-β and oral tolerization using MBP greatly reduces the clinical score of the induced EAE (see 8B). This result is unexpected, especially when contrasted to the effect of a combination of intraperitoneal mock rat IFN-β and oral tolerization using MBP. The mock rat IFN-β/MBP combination is at best of intermediate effect as compared to the PBS control treatment and oral tolerization with MBP alone. Thus, the combination treatment of intraperitoneal IFN-β and oral tolerization with MBP shows a synergistic suppressive effect on the clinical score of the EAE seen. In other words, the interferon potentiates the tolerizing ability of the bystander. These treatments clearly have a much greater effect on clinical score when used in conjunction compared to the effect achieved with either treatment alone. The synergistic interaction is best represented by Figure 5, where the difference between the third bar (GP-MBP(PO)+RAT IFN(IP)) from the top of the graph and the sixth bar (PBS(PO)) is greater than the addition of the differences between the fourth (RAT IFN(IP)) and sixth bar (PBS(PO)) and the fifth (GP-MBP(PO)) and sixth bar (PBS(PO)). The third bar is also strikingly smaller

than the first bar, a direct control of the combination treatment.

This synergistic or potentiating effect is further illustrated in the data of Figure 6. Again, the effect on the clinical score of the combination treatment is greater than the addition of the effects of each treatment alone.

Similar results have been achieved with the treatment of EAE in mice when PLP is used as the bystander antigen instead of MBP. As seen in Figure 7, treatment with either bovine MBP or bovine PLP suppressed the clinical score of the immunized animals (see B and C). Intraperitoneal administration of mouse IFN-β alone had no effect on the disease (see A).

However, a combination of the IFN-β treatment with oral tolerization using either bovine MBP or bovine PLP showed a significant reduction in clinical score (see B and C, respectively). The combination treatment appears to decrease the duration of EAE in mice (note return to clinical score of 0, especially for combination with bovine MBP (B)). Therefore, the synergistic suppressive effect seen in rats is also seen in the mouse EAE system. This effect is better illustrated in the bar graph of Figure 8. The difference between the first and sixth bar is clearly greater than the difference between the second and sixth bar or the difference between the fifth and sixth bar, indicating the combination therapy is more effective than either the oral tolerization or IFN-β treatment alone. Further, if the effect on clinical score of bovine PLP alone is added to the effect on clinical score of mouse IFN-β alone, the total reduction is still less than the reduction of clinical score achieved by the combination treatment. Thus, the combination has a synergistic effect on clinical score, as compared to the effects of each treatment method alone.

The result of experiments with oral Type I IFN in combination with oral (bystander) toleration therapy also show synergy, or potentiation.

Finally, experiments with orally-administered IFN-β alone in NOD mouse show IFN-β to be as effective in suppressing disease as orally-administered insulin.

The following examples are illustrative of the present invention and do not limit the scope of the invention.

In the experiments described below the following materials and methods were used.

Animals. Female Lewis rats 6-8 weeks of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). SJL/J mice, 8 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME. Animals were maintained on standard laboratory chow and water ad libitum. Animals were maintained in accordance with the guidelines for the Committee on Care of Laboratory Animals of the Laboratory Research Council (Pub. #DHEW:NIH, 85-23, revised 1985).

Antigens and Reagents. Guinea pig MBP was purified from brain tissue by the modified method of Deibler et al. (Prep. Biochem. 2:139, 1972). Protein content and purity were monitored by gel electrophoresis and amino acid analysis. Concanavalin A and histone were obtained from Sigma (St. Louis, MO). Peptides were synthesized in the peptide facility of the Center for Neurologic Disease, Brigham and Women's Hospital, and purified on HPLC. The amino acid sequences of the peptides synthesized are: 21-40, MDHARHGFLPRHRDTGILDS (immunosuppressive epitope region when orally administered to rats); 71-90, SLPQKSQRSQDENPVVHF (immunodominant encephalitogenic region in rats); 151-170, GTLSKIFKLGGRDSRS.

Interferons. Rat, mock rat, and mouse IFN-α/β were obtained from Cytimmune, Lee Biomolecular Research, San Diego, CA. Consensus interferon (CON 1) is available from Amgen, Inc.

Induction of Tolerance. For oral tolerance or active suppression, rats were fed 1 mg of MBP dissolved in 1 ml PBS, or PBS alone, by gastric intubation with a 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times at intervals of 2-3 days with the last feeding two days before immunization.

Induction of EAE. For actively induced disease, Lewis rats were immunized in the left foot pad with 25 μg of guinea pig MBP in 50 μl of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 4 mg/ml of *Mycobacterium tuberculosis* (Difco). For adoptively transferred

EAE, an MBP active T cell line was established from rats immunized with MBP in CFA, raised and maintained according to the method of Ben-Nun et al. (Euro. J. Immunol. 11:195, 1982). Encephalitogenic cells were collected after activation by culture with Concanavalin A (ConA) (2 μm/ml) using irradiated thymocytes from immunized animals as antigen presenting cells (APCs). Cells were harvested from cultures via a ficol hypaque gradient (Hypaque 1077, Sigma) and washed twice in PBS prior to transfer. $5x10^6$ encephalitogenic cells were injected intraperitoneally in 0.1 ml PBS into irradiated (750 rads, 24 hrs. earlier), recipient rats. Cell viability of both modulator and encephalitogenic cells was determined by trypan blue exclusion and was greater than 90%.

Clinical evaluation. Animals were evaluated in a blind fashion every day for evidence of EAE. Clinical severity of EAE was scored as follows: 0, no disease; 1 limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; and 5 death. Duration of disease was measured by counting the total number of days from disease onset (usually days 10 or 11 after active immunization and 3-5 days after adoptive transfer of disease) until complete recovery for each animal.

Delayed type hypersensitivity (DTH) testing. DTH was tested by injecting 25 μg of MBP in PBS subcutaneously in the ear. Thickness was measured by a blinded observer, before and 48 hours after challenge, using micrometer calipers (Mitutoyo, Japan). The difference of ear thickness before and after challenge was recorded for each animal, and the result was expressed as the mean for each experimental group ± SEM.

Histology. Histologic analysis of pathological changes was performed in rats with adoptively transferred EAE. Spinal cords were removed on day 15 after adoptive transfer (or disease induction) and fixed with 10% neutral buffered formalin. Paraffin sections were prepared and stained with Luxol fast blue-hematoxylin and eosin, by standard procedures (Sobel et al., J. Immunol. 132:2393, 1984). Spinal cord tissue was sampled in an identical manner for each animal and numbers of inflammatory foci per section (clusters of >20 or more

aggregated inflammatory cells), in parenchyma and meninges were scored in a blinded fashion (Sobel et al., <u>supra</u>).

<u>Statistical analysis</u>. Clinical scales were analyzed with a two-tailed Wilcoxon rank sum test for score samples, chi square analysis was used in comparing the incidence of disease between groups, and comparison of means was performed by using the Student's t-test. For individual experiments, 5 animals were generally used per group.

<u>EXAMPLE 1</u>: <u>Assay for TGF-β Induction</u>

<u>Measurement Of TGF-β Activity In Serum-Free Culture Supernatants</u>. Serum free culture supernatants were collected from the antigen-tolerized animals as previously described (Kehri, et al. <u>J. Exp.Med.163</u>: 1037-1050, 1986; Wahl, et al. <u>J.Immunol.145</u>: 2514-2419,1990). Briefly, modulator cells were first cultured for 8 hours with the antigen (50 μl/ml) in proliferation medium. Thereafter cells were washed three times and resuspended in serum-free medium for the remainder of the 72 hour culture, collected, then frozen until assayed. Determination of TGF-β content and isoform type in supernatants was performed using a mink lung epithelial cell line (American Type Culture Collection, Bethesda, MD #CCL-64) according to Danielpour et al. (Danielpour, D., et al. <u>J. Cell. Physiol.</u> 138: 79-86,1989).), and confirmed by a Sandwich Enzyme Linked Immunosorbent Assay (SELISA) assay as previously described (Danielpour et al. <u>Growth Factors 2</u>: 61-71,1989). The percent active TGF-β was determined by assay without prior acid activation of the samples.

This assay can be adapted to test any antigen which is a candidate for use as a bystander. Those antigens, antigen fragments and/or amounts of antigen which produce the highest concentration of TGF-β as measured by this assay can be considered those antigens and/or amounts most suitable for use in the treatment method of the present invention. Alternatively, a transwell culture system, described below, can be used to indicate the level of TGF-β which is being produced. This culture system measures the production of TGF-β as a function of suppression of cell proliferation.

Transwell Cultures. A dual chamber transwell culture system (Costar, Cambridge, MA), which is 24.5 mm in diameter and consists of two compartments separated by a semi-permeable polycarbonate membrane, with a pore size of 0.4 μm, was used. The two chambers are 1 mm apart, allowing cells to be coincubated in close proximity without direct cell-to-cell contact. To measure in vitro suppression of proliferative responses in transwell cultures, 5 x 10^4 antigen line cells, raised and maintained for example, as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195, 1981), were cultured with 10^6 irradiated (2,500 rad) thymocytes, in 600 μl of proliferation media in the lower well. Spleen cells from orally tolerized rats or controls (fed BSA) were added to the upper well (5 x 10^5 cells in 200 μl). Spleen cells were removed 7-14 days after the last feeding, and a single cell suspension was prepared by pressing the spleens through a stainless steel mesh. The antigen (50 μg/ml) is added in a volume of 20 μl. Because modulator cells are separated from responder cells by a semi-permeable membrane, they do not require irradiation. In some experiments, modulator cells were added in the lower well together with responder cells, and in these instances modulator cells were irradiated (1,250 rad) immediately before being placed in culture. Proliferation media consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 x 10^5 M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1 % HEPES buffer, 1% nonessential amino acids, and 1% autologous serum. Each transwell was performed in quadruplicate. The transwells were incubated at 37°C in a humidified 6% CO_2 and 94% air atmosphere for 72 hours. After 54 hours of culture, each lower well was pulsed with 4 μCi of [^{3}H]thymidine and at 72 hours split and reseeded to three wells in a round-bottomed 96-well plate (Costar) for harvesting onto fiberglass filters and counting using standard liquid scintillation techniques. Percent suppression = 100 x (1 - Δ cpm responders cultured with modulators/Δ cpm of responders).

EXAMPLE 2: Identification of Immunosuppressive Epitopes of Guinea Pig MBP by Oral Route

To investigate the mechanism of oral vs. IV tolerance, MBP peptides encompassing both encephalitogenic and non-encephalitogenic regions of MBP were administered both orally and intravenously prior to immunization for actively induced disease. MBP peptide 71-90 of guinea pig MBP is encephalitogenic in Lewis rats (Swanborg et al., J. Immunol. 114:191, 1975). As shown in Figure 1B, suppression of EAE via IV tolerization only occurred with whole MBP and encephalitogenic peptide 71-90, but not with guinea pig MBP peptide 21-40. Oral tolerization with 21-40, however, was effective in suppressing EAE (Fig. 1A). Guinea-pig peptide 21-40 was chosen as previous experiments have demonstrated that it caused spleen cells of rats orally tolerized to whole MBP to release TGF-β. Miller, A. et al. FASEB 6:1686, 1992. Control guinea pig MBP peptide 131-150 did not suppress when administered either orally or intravenously (Fig. 1A). Of note is that in addition to suppressing via the IV route, encephalitogenic MBP peptide 71-90 also suppressed when given orally. This result indicates that peptides derived from the immunodominant domain of a given MBP towards a given host can suppress T-cell function when they are orally or intravenously administrated, but do so by different mechanisms depending on the route and protocol of administration.

The results of these experiments show that there are basic differences in the mechanism of suppression of EAE between orally and parenterally (e.g. intravenously) administered MBP. The results suggest that orally administered antigen acts predominantly via the generation of active suppression, whereas parenterally administered antigen acts via clonal anergy. Specifically supporting this conclusion is the inability of spleen cells from IV tolerized animals to suppress adoptively-transferred EAE. Additionally, different fragments of MBP displayed different abilities to suppressing EAF depending on the routes of administration (see, for example, Figure 1).

The transwell System of Example 1 above was used to identify the epitopes present on guinea pig MBP which induce the release of TGF-β from suppressor T-cells.

The disease-inducing fragments (autoimmune response epitopes) of MBP were first confirmed as follows: Overlapping peptides of guinea pig MBP were obtained from commercial sources or synthesized in accordance with well-known techniques, specifically using a commercial peptide synthesis apparatus (from Applied Biosystems) and following the manufacturer's instructions. Whole MBP was then fed to rats and lymph node cells from the orally tolerized animals were triggered with the MBP-peptides. The ability of the triggered cells to induce killer T-cells was then quantitatively determined by a proliferation assay, as described in Example 4, and by testing the ability of the proliferating cells to transfer the disease.

A peptide spanning residues 71-90 of guinea pig MBP was by far the most efficient inducer of killer T-cells and therefore the most potent disease-promoting fragment of MBP. This region of guinea pig MBP therefore corresponds to the immunodominant epitope of the protein.

When spleen cells obtained from animals fed MBP and immunized with MBP/CFA (as described above in Example 1) were co-cultured in the transwell system with spleen cells isolated from OVA-fed animals, peptides corresponding to guinea pig MBP amino acid residues 21-40, 51-70 and 101-120 added to the modulator well were all capable of triggering suppression of proliferation of the OVA-fed line. But, contrary to other experiments with different animals, the immunodominant epitope of guinea pig MBP in rats, (corresponding to amino acid residue nos. 71-90) was ineffective in triggering suppression in the transwell system. Peptides corresponding to guinea pig MBP residue nos. 151-170 and 161-178 inhibited proliferation of the OVA (responder) line but this effect was non-specific, and may have been due to toxicity induced <u>in vitro</u> by these peptides, as these same peptides inhibited proliferation of spleen cells isolated from OVA-fed animals when co-cultured with control (non-MBP-fed) modulator cells (data not shown).

Such a system can be easily adapted by one of ordinary skill such that peptides of the antigen of interest which are effective in the present treatment method can be identified.

EXAMPLE 3: **Oral Tolerance Using Bovine-PLP or Mouse MBP**

In order to demonstrate bystander suppression, groups of 5-6 female, 7 week old, SJL/J mice (Jackson Labs, Bar Harbor, ME) were immunized with PLP peptide 140-160 on days 0 and 7 and received the following treatments:

GROUPS

1. Fed Histone (0.25 mg/mouse)
2. Fed Mouse MBP (0.25 mg/mouse)
3. Fed Bovine PLP (0.25 mg/mouse) (autoantigen suppression)

Each group was treated every other day for 7 days. In the intravenous group, the material was injected into the eye plexus. The PLP peptide used was the disease inducing fragment 140-160 of bovine PLP. This peptide has the amino acid sequence $COOH-PLAYTIGVFKDPHGLWKGLCNH_2$, representing the foregoing amino acid residues.

As shown in Figure 2, both mouse MBP and bovine PLP were equally effective in down-regulating PLP-peptide-induced EAE when orally administered. A non-specific protein, histone, was ineffective in suppressing EAE when administered orally. Thus, a bystander antigen, in this case mouse MBP, effectively suppressed EAE when orally administered to animals induced for EAE with bovine PLP.

The effects of feeding various peptides to Lewis rats induced for EAE by guinea pig MBP residue nos. 71-90 (the major immunodominant epitope of guinea pig MBP as shown in Example 1 above) were also studied.

EAE was induced by immunizing with 0.25 mg of guinea pig MBP amino acid residue nos 71-90 in Complete Freund's Adjuvant and the effect of feeding various guinea pig MBP peptides on EAE was examined.

As shown in Fig. 3, orally administered whole guinea pig MBP and a 21-40 guinea pig peptide were equally effective in downregulating EAE induced by guinea pig MBP 71-90 as was orally administered 71-90 itself. Guinea pig MBP peptide 131-150 was ineffective in conferring tolerance. Peptides were also fed with STI which prevents their breakdown by gastric juices and enhances their biological effect. DTH responses to whole MBP were suppressed by feeding MBP or any one of the MBP-peptides 21-40, or 71-90. However, DTH responses to guinea pig MBP peptide 71-90 were only suppressed by feeding either whole MBP or guinea pig peptide 71-90 and were not affected by guinea pig MBP peptide 21-40 (Fig. 3). This is consistent with the conclusion that MBP fragment 71-90 does not participate in bystander suppression when fed to mice in which disease had been induced with peptide 71-90.

EXAMPLE 4: Suppression of EAE in Rats with a combination of Oral Tolerization using Guinea pig (GP) MBP and Intraperitoneal IFN-β

EAE was induced in thirty female Lewis rats, weighing between 175 and 200g, by immunizing on day 0 with 100μl of an emulsion of 0.25 mg of GP MBP and 100mg of Mycobacterium tuberculosis (Mt), as an adjuvant. These rats were divided into five groups and were given the following treatments on days 10, 12, and 14:

Groups

1. Fed PBS (1ml/rat)
2. Fed GP MBP (1mg/rat)
3. Injected rat IFN-α/β (15,000 units/rat)
4. Fed GP MBP (1mg/rat) + injected rat IFN-α/β (150,000 units/rat)
5. Fed GP MBP (1mg/rat) + injected mock IFN-β (.20 IRU/rat
6. Injected mock rat IFN-β (.20 IRU/rat)

All injections were done intraperitoneally. Following treatment, clinical evaluations, as described above, were recorded daily for each rat, with the average clinical score for each group providing the data point for each day post-immunization.

As shown in Figures 4 and 5, oral tolerization with MBP had its expected suppressive effect on clinical score (see Figure 4, A-D). At the amount administered, IFN-α/β alone had very little effect on the disease, and is comparable to both the PBS controls as well as the mock IFN-β control (see Figure 4, C and D). However, the combination of feeding GP MBP and intraperitoneal injection of IFN-β significantly reduces the clinical score seen in the treated rats. This is especially significant given the apparent ability of the mock IFN-β preparation to counter the ability of MBP to suppress EAE (see Figure 4, D). In summary, the mean maximum clinical score in this experiment was:

PBS	α/β IFN	MBP	α/β IFN + MBP	mock IFN	mock IFN + MBP
3.0	2.4	2.0	0.6	2.6	2.4

Figure 6 relates similar data from an experiment which was done on female Lewis rats (150-200g) fed with 1mg of MBP, and.or injected with 150,000 units rat α/β-IFN on days -4, -2 and 0 and immunized with 25 mg GP-MBP.

The combination of oral GP MBP with intraperitoneal IFN-β has a synergistic suppressive effect of EAE in rats. This assertion is based on the comparison between the level of suppression seen with each treatment separately, and the superadditive level of suppression seen with the combination treatment. The suppression seen with the combination is in excess of the addition of the two levels of suppression achieved with each separate treatment. The above results were confirmed by delayed-type hypersensitivity experiments. In addition, measurements of the in vitro production of cytokines by lymphocytes from fed animals in response to specific antigen in culture, showed that the synergistic effect may be related to enhanced production of TGFβ and IL4 or IL10. βIFN thus acts as a synergist to enhance oral tolerance to EAE.

EXAMPLE 5: Suppression of EAE in Mice with a Combination of Oral Tolerization using MBP or PLP and Intraperitoneal IFN-β

EAE was induced in 35 SJL/J, 8 week old, female mice by immunizing on day 0 and day 7 with 0.2 ml of an emulsion containing 200mg of bovine PLP and 200 mg of Mt. These mice were divided into 7 groups and received the following treatments on days 5, 8, and 10:

GROUPS

1. Fed hen egg lysozyme (HEL) (0.25mg/mouse)
2. Injected mouse IFN-β (69,000 units/mouse)
3. Fed bovine MBP (0.25 mg/mouse)
4. Fed bovine MBP (0.25 mg/mouse) + injected mouse IFN-β (69,000 units/mouse)
5. Fed bovine PLP (0.25 mg/mouse)
6. Fed bovine PLP (0.25 mg/mouse) + injected mouse IFN-β (69,000 units/mouse)

All injections were done intraperitoneally. Following treatment, clinical evaluations, as described above, were recorded daily for each mouse, with the average clinical score for each group providing the data point for each day post-immunization.

The combination of oral bovine MBP with intraperitoneal IFN-β and the combination of oral bovine PLP with intraperitoneal IFN-β both have a synergistic suppressive effect of EAE in mice (see Figures 7 and 8). This assertion is based on the comparison between the level of suppression seen with each treatment separately, and the superadditive level of suppression seen with the combination treatment. The superadditive effect attributable to the combination is the excess of the sum of the effect achieved with each separate treatment.

EXAMPLE 6: Synergy of Oral IFN-β and Oral MBP in Rat EAE Suppression

Female Lewis rats (150 to 200 g) were fed with 1 mg of Myelin Basic Protein (MBP), varying doses of rat α/β Interferon, or a combination of MBP and interferon. Oral proteins were given a total of seven times every other day, 4 preimmunization and 3 postimmunization with 25 μg gpMBP for the induction of EAE. Animals were scored for signs of paralysis beginning on day 9 on a scale of 0 to 5.

The results were as follows:

GROUPS FED	INCIDENCE	DAY OF ONSET	MEAN MAX SCORE
HEL 1.0 mg	5/5	12.0	2.0
βIFN 20000 U	4/5	13	1.0
β IFN 10000 U	5/5	12	1.4
β IFN 5000 U	5/5	12.6	1.0
gpMBP 1.0 mg	5/5	12.4	1.0
gpMBP 1.0 mg +β IFN 20000 U	4/5	13.2	0.8

Example 7: **Synergy of Oral β-IFN and Oral MPP in Mouse EAE Suppression**

Female SJL mice were fed with 0.25 mg the bovine brain protein Myelin Basic Protein (MBP) with or without 5000 Units of murine β-lnterferon three times prior to immunization with 200 μg PLP and 200 μg MT for the induction of EAE. Animals were scored for signs of paralysis beginning on day 9 on a scale of 0 to 5.

The results were as follows:

GROUPS FED	INCIDENCE	DAY OF ONSET	MEAN MAX SCORE
HEL 0.25 mg	5/5	15.8	2.2
βIFN 5000 U	4/5	17.2	2.1
gpMBP 0.25 mg	4/5	18.8	1.6
gpMBP 0.25 mg +β IFN 5000 U	2/5	17.5	0.9

Example 8: **Effect of Oral β-lnterferon on the Induction of Adjuvant Arthritis in Rats**

Female Lewis Rats weighing 120-140 grams were fed Collagen Type II (CII) at the indicated doses and/or 5000 Units of βInterferon every other day starting on day -10 before

immunization. On day 0, animals were injected with 1 mg/ 0.1 ml of Mycobacterium tuberculosis (MT) id. Beginning on day +10, animals were scored for signs of arthritis on a scale of 0 to 4. The arthritis score for each animal was the sum of the score, for each of the four paws.

The results are in Fig. 9.

Example 9: Oral IFN-β in Suppression of NOD Diabetes

Groups of NOD mice (3 animals per group) were treated as follows:

1. control
2. ovalbumin fed (1mg/feeding/mouse)
3. equine insulin fed (1mg/feeding/mouse)
4. Mouse IFN-β (5000 u/feeding/mouse)

All mice were fed 10 times on alternate days. About 3 weeks after the experiment the following parameters were assessed = insulitis, CD5 T-cells, CD4 T-cells, CD8 T-cells, macrophages, and various cytokines. The results are presented below:

Group	Unfed	Fed Ovalbumin	Fed Insulin	Fed β-IFN Alone
Morphology	massive peri-islet and some intra- islet MNC infiltrates	massive peri-islet and some intra- islet MNC infiltrates	mild to moderate peri-islet MNC infiltrates	very little or no insulitis
CD5 T-cells	>75% MNC	>75% MNC	>75% MNC	very few + cells
CD4 T-cells subset	dense infiltrates including in islets, moderate number of CD8+ cells	>75% MNC	>75% MNC	very few positive cells
CD8 T-cell subset	dense infiltrates, though islets mostly negative	dense though very small number of CD4+, islets mostly negative	some positive but very small number of CD4+ cells	very few positive cells
Macrophages (F4/80)	about -5% MNC including intra-islet cells	about -5% MNC including intra-islet cells	small number of adven-titial and peri-islet cells	small number of adven-titial and peri-islet cells

Group	Unfed	Fed Ovalbumin	Fed Insulin	Fed β-IFN Alone
IL-2R	small number of peri-islet MNC	5-10% MNC including intra-islets	very few positive cells	1-2 cells/ section
IL-2	small numbers of peri- and intra-islet MNC	small numbers of peri- and intra-islet MNC	negative	negative
IL-4	negative	negative	small number of peri- and intra-islet MNC	small number of peri- and intra-islet MNC
IL-7	negative	negative	negative	negative
IL-10	negative	only a few cells/ section	small number of peri- and intra-islet MNC	only a few cells/ section
IFN-gamma	>50% MNC adjacent or in islets	>50% MNC adjacent or in islet	negative	negative
IFN-alpha	>50% MNC adjacent or in islets	>50% MNC adjacent or in islets	negative	negative

WHAT IS CLAIMED:

1. A method for treating a mammal diagnosed with a T-cell mediated or T-cell dependent autoimmune disease the method comprising the step of:

orally or enterally administering to said mammal an amount of (i) a bystander antigen in conjunction with an amount of (ii) a polypeptide having Type I interferon activity, the amounts of (i) and (ii) being effective in combination in suppressing autoimmune response in said mammal.

2. The method of claim 1 wherein the amounts of (i) and (ii) are synergistically effective in suppressing said response in combination compared to the sum of the suppressive effects achieved by administering each of (i) and (ii) alone.

3. The method of claim 1 wherein said polypeptide is IFN-β and is administered parenterally.

4. The method of claim 1 wherein said polypeptide is IFN-β and is administered orally.

5. The method of claim 1 wherein said mammal is a rodent and said disease is a rodent model for multiple sclerosis.

6. The method of claim 1 wherein said mammal is a human and said disease is multiple sclerosis.

7. The method of claim 5 wherein said bystander antigen is selected from the group consisting of myelin basic protein (MBP), proteolipid protein (PLP), fragments thereof and combinations of at least two of the foregoing.

8. The method of claim 6 wherein said bystander antigen is selected from the group consisting of myelin basic protein (MBP), proteolipid protein (PLP), fragments thereof and combinations of at least two of the foregoing.

9. The method of claim 7 wherein said IFN-β polypeptide is derived from the same species as said mammal.

10. The method of claim 8 wherein said IFN-β polypeptide is derived from human IFN-β.

11. The method of claim 1 wherein said disease is selected from the group consisting of rheumatoid arthritis and animal models therefor and said bystander antigen is selected from the group consisting of Type I collagen, Type II collagen, Type III collagen, fragments thereof and combinations of two or more of the foregoing.

12. The method of claim 1 wherein said disease is selected from the group consisting of Type I diabetes and animal models therefor and said bystander antigen is selected from the group consisting of glucagon, insulin, fragments thereof, and combinations of two or more of the foregoing.

13. The method of claim 1 wherein said disease is selected from the group consisting of uveoretinitis and animal models therefor and said bystander antigen is selected from the group consisting of S-antigen, interphotoreceptor retinoid binding protein (IRBP), fragments thereof, and combinations of two or more of the foregoing.

FIG. 1A

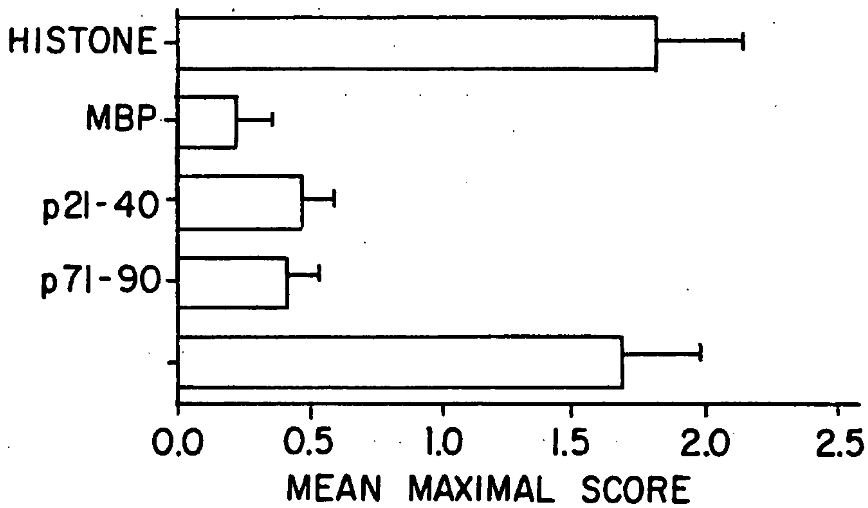

HISTONE
MBP
p21-40
p71-90
0.0
0.5
1.0
1.5
2.0
2.5
MEAN MAXIMAL SCORE

FIG. 1B

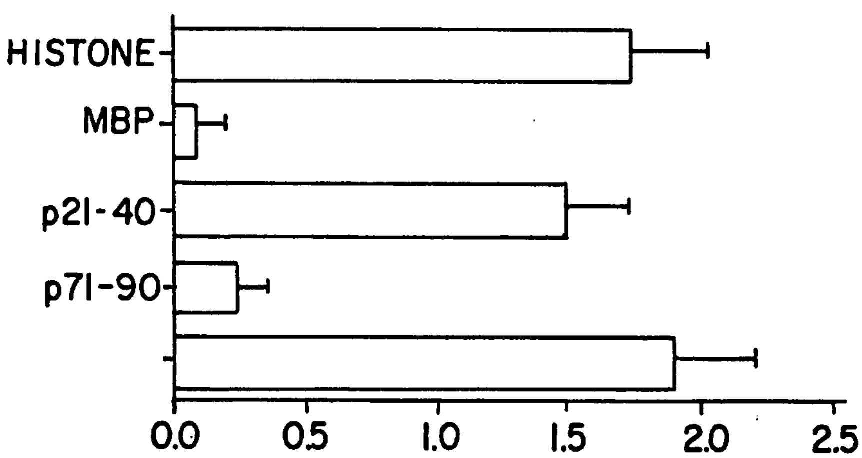

HISTONE
MBP
p21-40
p71-90
0.0
0.5
1.0
1.5
2.0
2.5

FIG. 2A

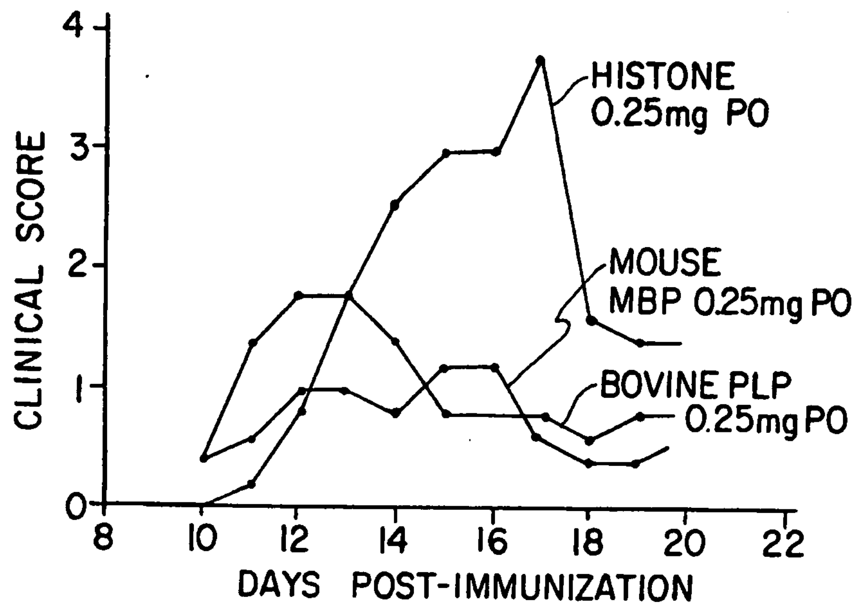

HISTONE 0.25mg PO
MOUSE MBP 0.25mg PO
BOVINE PLP 0.25mg PO
CLINICAL SCORE
DAYS POST-IMMUNIZATION
0
1
2
3
4
8
10
12
14
16
18
20
22

FIG. 2B

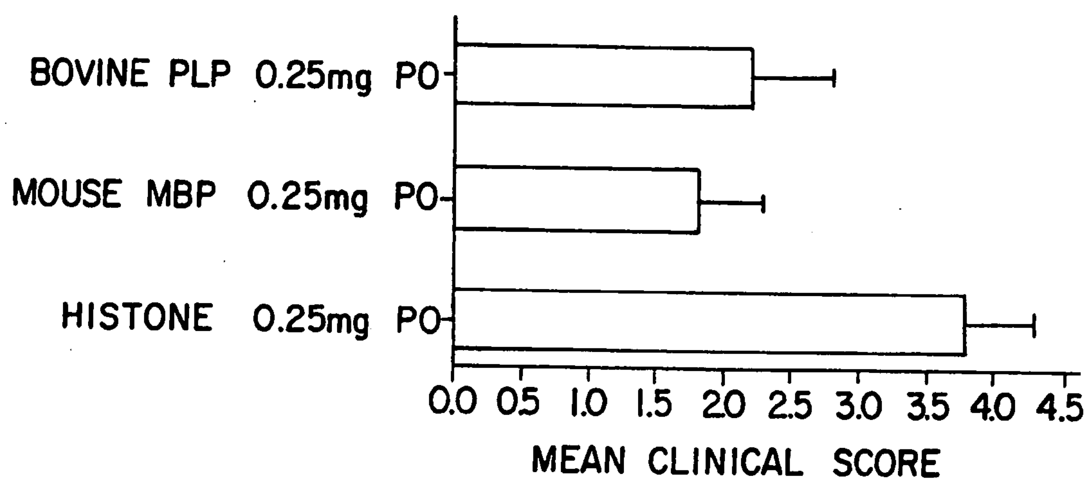

BOVINE PLP 0.25mg PO
MOUSE MBP 0.25mg PO
HISTONE 0.25mg PO
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5
MEAN CLINICAL SCORE

FIG. 3

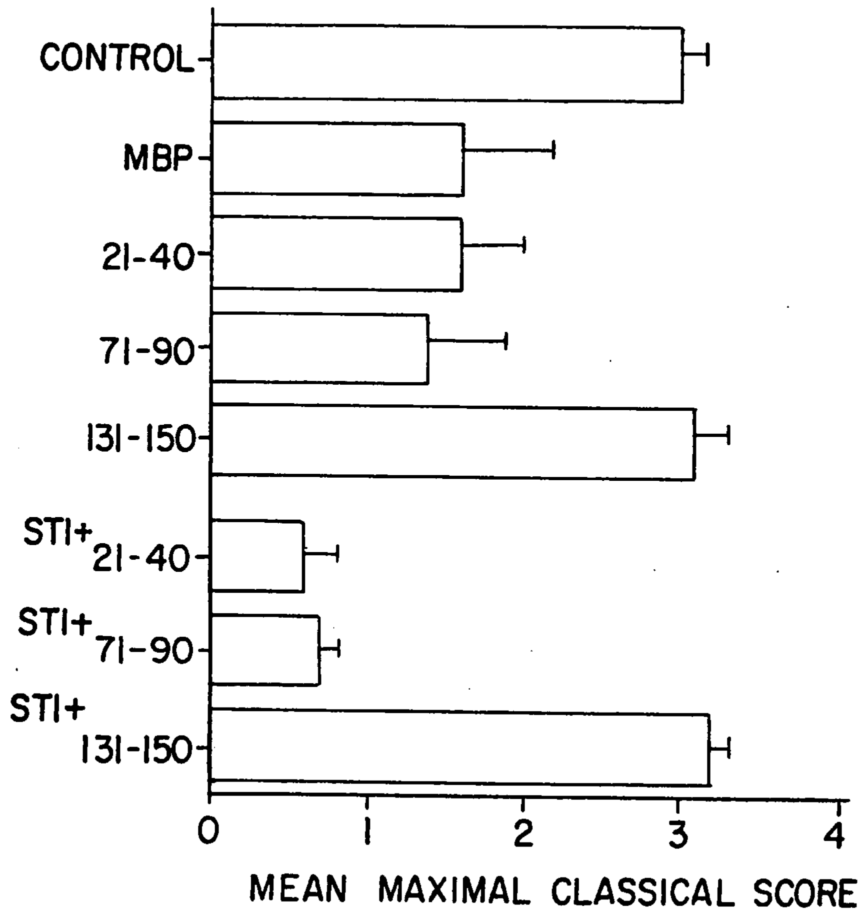

CONTROL
MBP
21-40
71-90
131-150
STI+ 21-40
STI+ 71-90
STI+ 131-150
0
1
2
3
4
MEAN MAXIMAL CLASSICAL SCORE

FIG. 4A

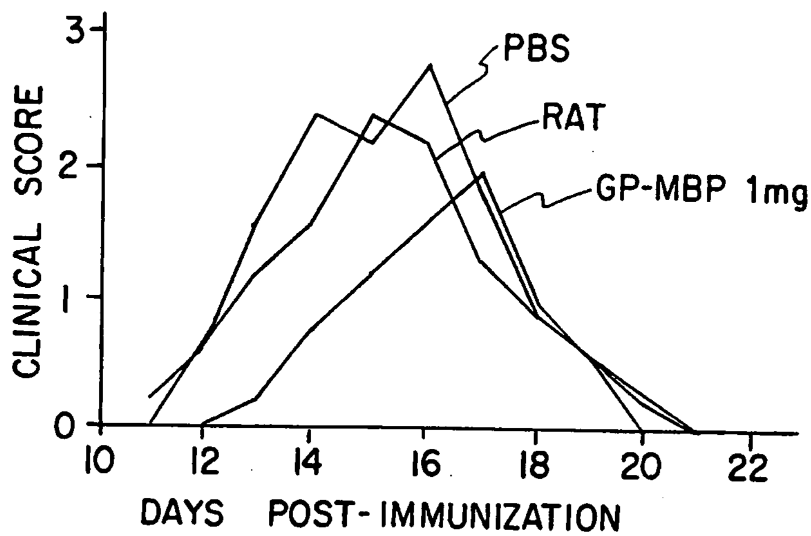

PBS
RAT
GP-MBP 1mg
CLINICAL SCORE
0
1
2
3
10
12
14
16
18
20
22
DAYS POST-IMMUNIZATION

FIG. 4B

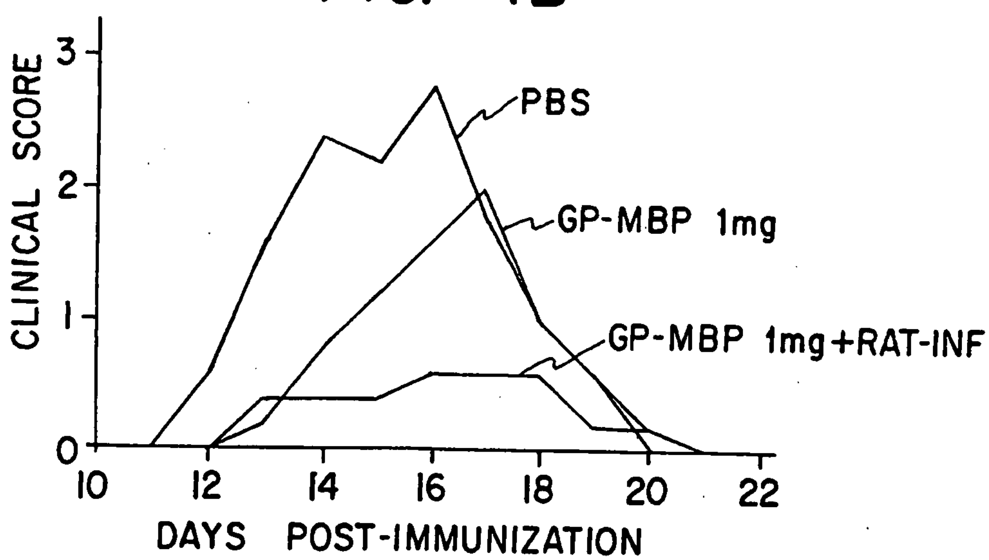

PBS
GP-MBP 1mg
GP-MBP 1mg+RAT-INF
CLINICAL SCORE
0
1
2
3
10
12
14
16
18
20
22
DAYS POST-IMMUNIZATION

FIG. 4C

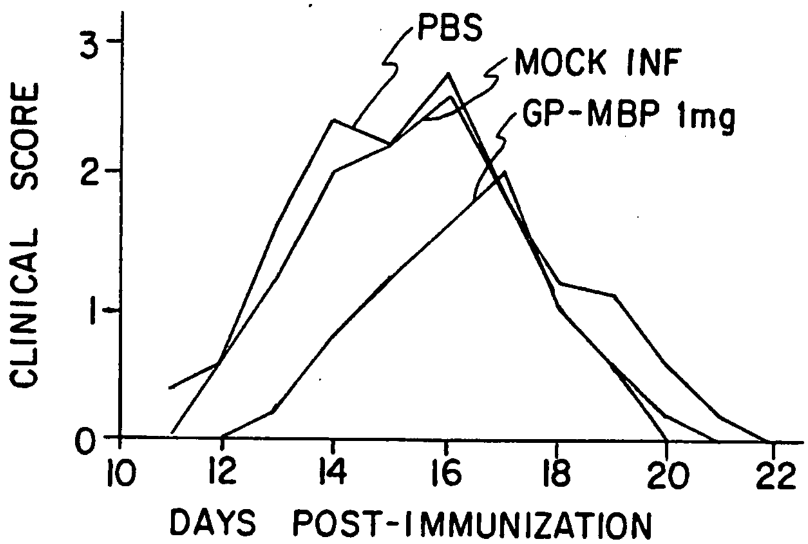

PBS
MOCK INF
GP-MBP 1mg
CLINICAL SCORE
0
1
2
3
10
12
14
16
18
20
22
DAYS POST-IMMUNIZATION

FIG. 4D

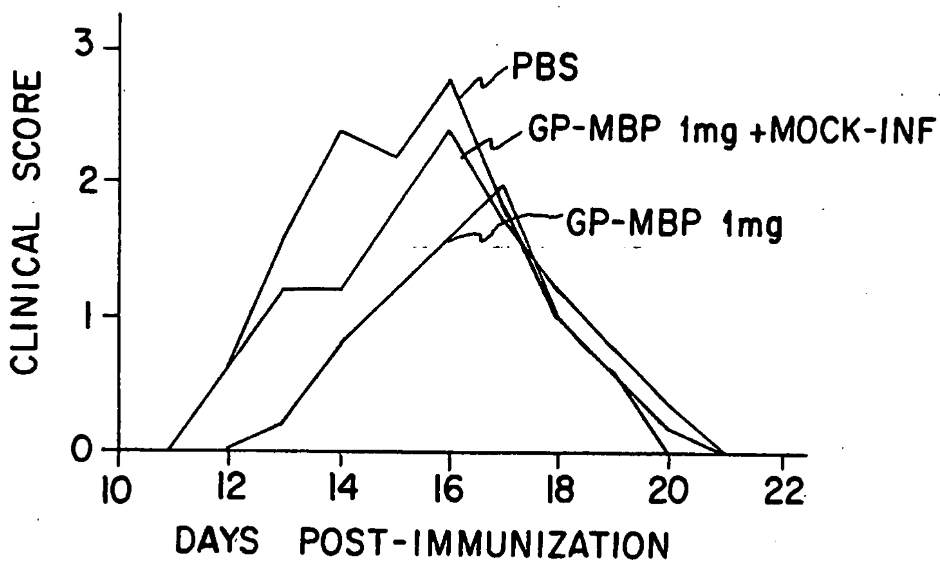

PBS
GP-MBP 1mg +MOCK-INF
GP-MBP 1mg
CLINICAL SCORE
0
1
2
3
10
12
14
16
18
20
22
DAYS POST-IMMUNIZATION

FIG. 5

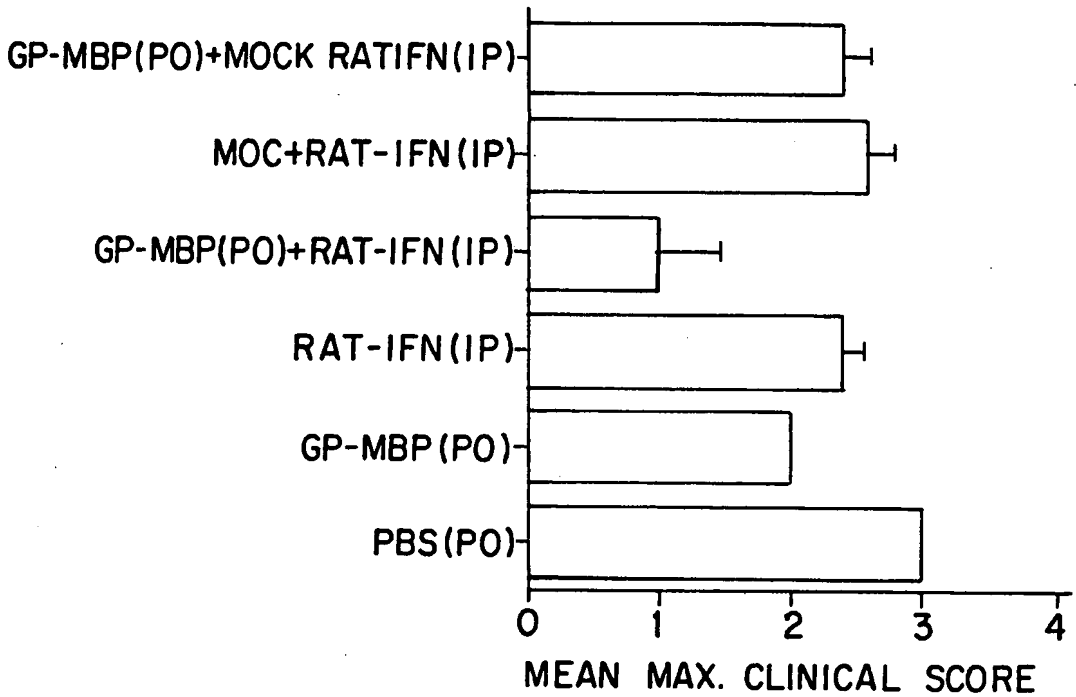

GP-MBP(PO)+MOCK RATIFN(IP)
MOC+RAT-IFN(IP)
GP-MBP(PO)+RAT-IFN(IP)
RAT-IFN(IP)
GP-MBP(PO)
PBS(PO)
0
1
2
3
4
MEAN MAX. CLINICAL SCORE

FIG. 6

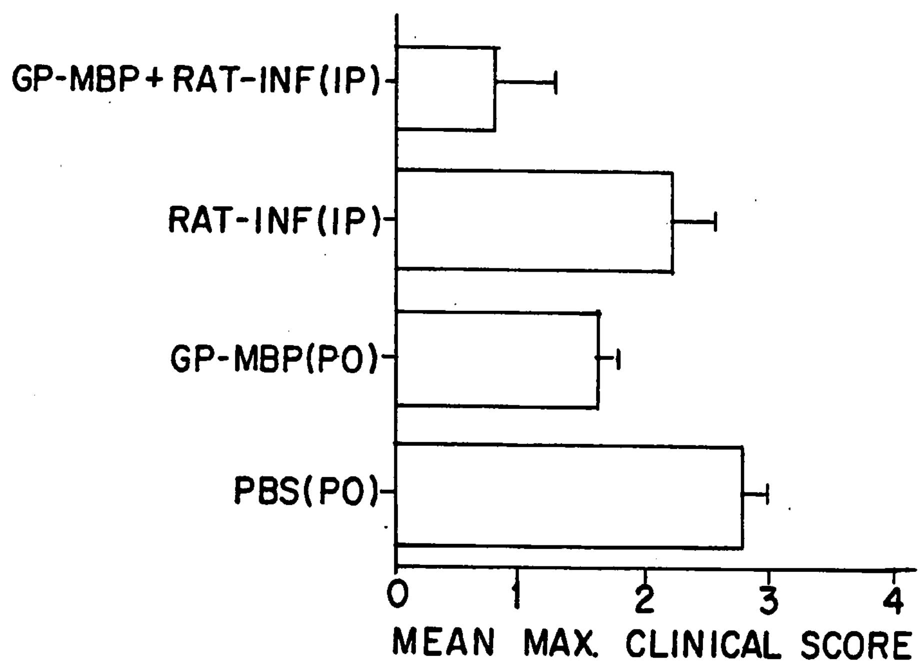

GP-MBP+RAT-INF(IP)
RAT-INF(IP)
GP-MBP(PO)
PBS(PO)
0
1
2
3
4
MEAN MAX. CLINICAL SCORE

FIG. 7A

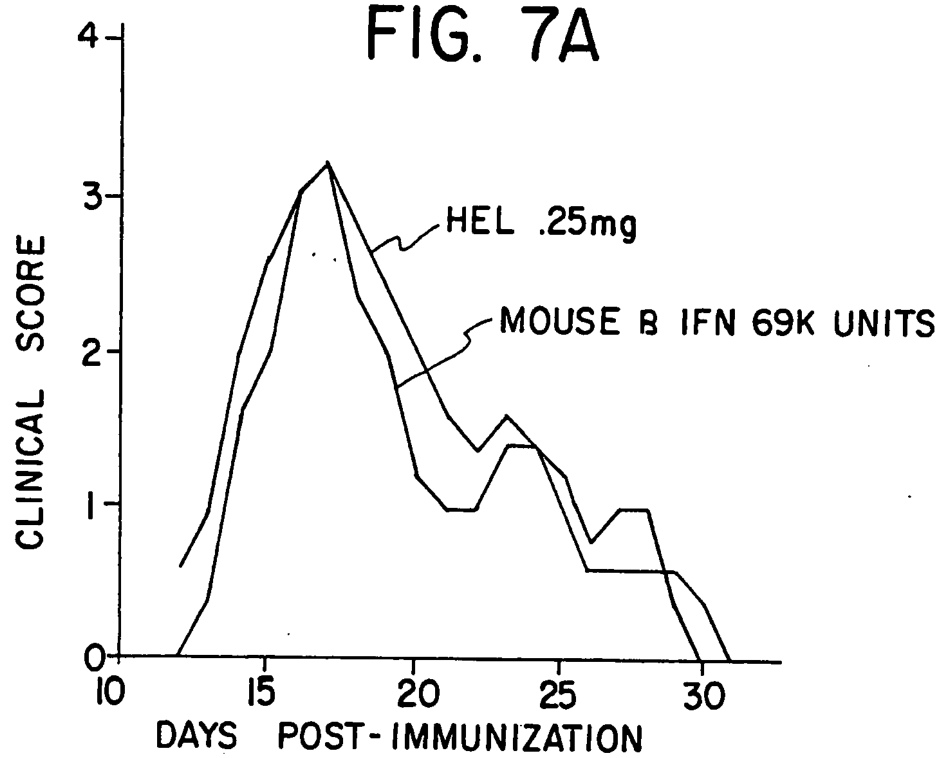

4
3
2
1
0
CLINICAL SCORE
HEL .25mg
MOUSE B IFN 69K UNITS
10
15
20
25
30
DAYS POST-IMMUNIZATION

FIG. 7B

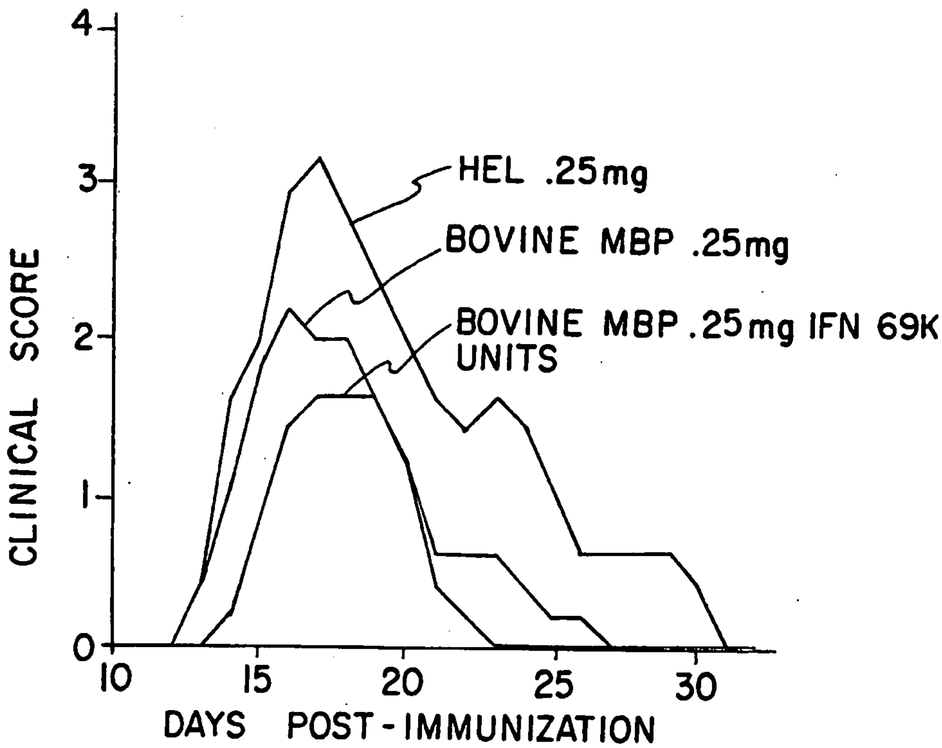

4
3
2
1
0
CLINICAL SCORE
HEL .25mg
BOVINE MBP .25mg
BOVINE MBP .25mg IFN 69K UNITS
10
15
20
25
30
DAYS POST-IMMUNIZATION

FIG. 7C

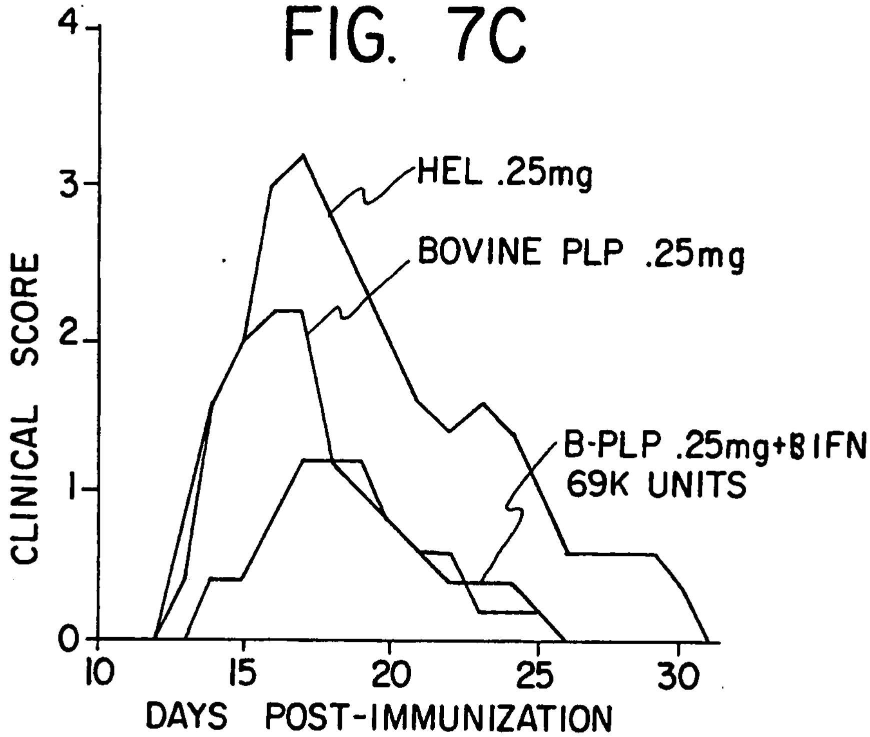

4
3
2
1
0
CLINICAL SCORE
HEL .25mg
BOVINE PLP .25mg
B-PLP .25mg+ß IFN
69K UNITS
10
15
20
25
30
DAYS POST-IMMUNIZATION

FIG. 8

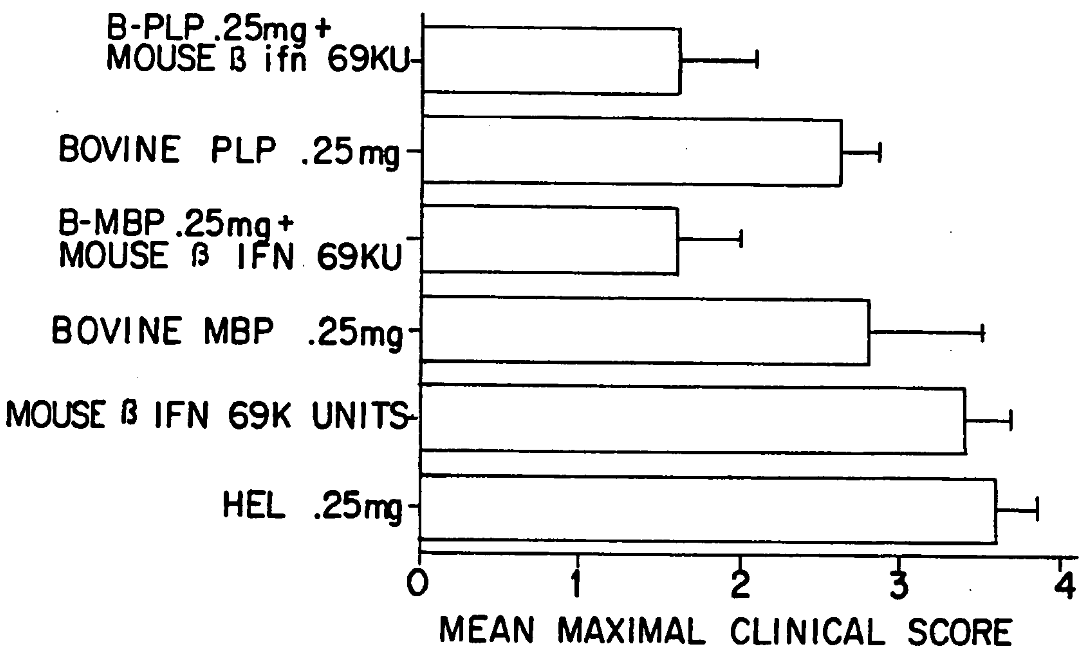
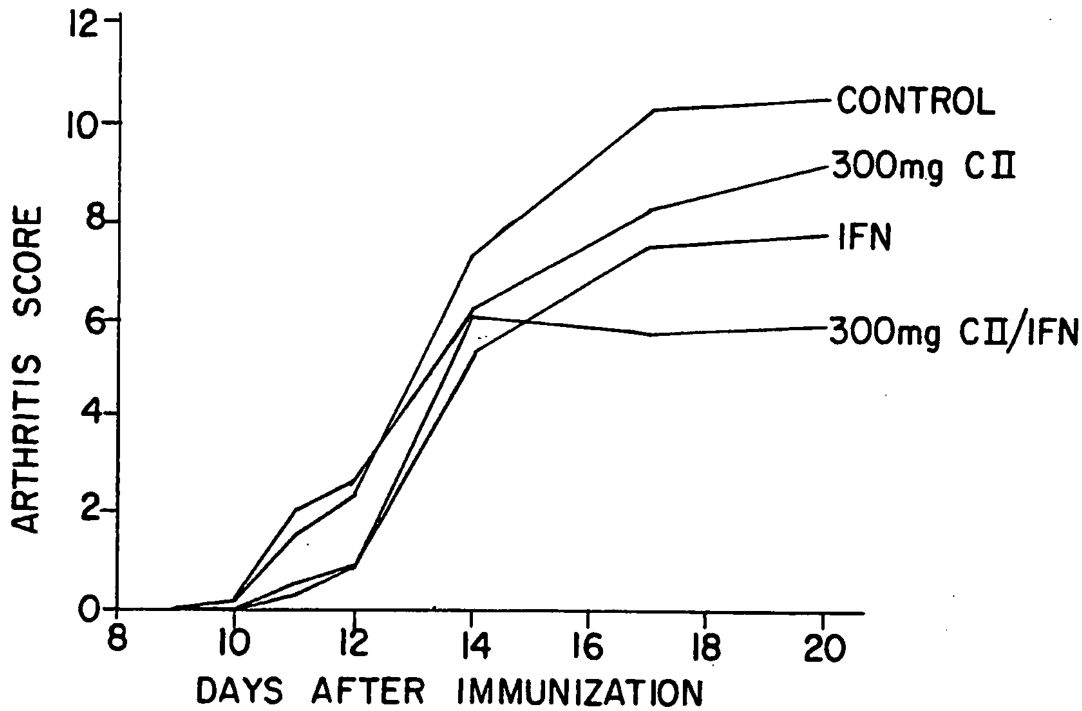

B-PLP .25mg +
MOUSE ß ifn 69KU
BOVINE PLP .25mg
B-MBP .25mg +
MOUSE ß IFN 69KU
BOVINE MBP .25mg
MOUSE ß IFN 69K UNITS
HEL .25mg
0
1
2
3
4
MEAN MAXIMAL CLINICAL SCORE

FIG. 9


12
10
8
6
4
2
0
ARTHRITIS SCORE
8
10
12
14
16
18
20
DAYS AFTER IMMUNIZATION
CONTROL
300mg CII
IFN
300mg CII/IFN

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04120

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/17, 38/21, 38/28, 38/39
US CL :424/85.4, 184.1; 514/2, 3, 8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.4, 184.1; 514/2, 3, 8; 530/303, 308, 350, 351, 356.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 18D, issued 07 April 1994, Nelson et al., "ß Interferon enhances oral tolerance to myelin proteins in experimental autoimmune encephalomyelitis," page 447, abstract V785, see Abstract.	1-10 ------- 11-13
X ---- Y	NEUROLOGY, Vol. 44, No. 4, issued April 1994, Al-Sabbagh et al., "Beta interferon enhances oral tolerance to MBP and PLP in experimental autoimmune encephalomyelitis," page A242, abstract 465P, see Abstract.	1-10 ------- 11-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 08 JULY 1995	Date of mailing of the international search report 18 JUL 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT D. BUDENS Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04120

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANNUAL REVIEWS OF IMMUNOLOGY, Vol. 12, issued 1994, Weiner et al., "Oral tolerance: Immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens," pages 809-837, see pages 817-818, 820, 824-831.	1-13
Y,P	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Vol. 91, No. 23, issued 08 November 1994, H. L. Weiner, "Oral tolerance," pages 10762-10765, see entire document.	1-13
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 142, No. 3, issued 01 February 1989, Lider et al., "Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein," pages 748-752, see entire document.	1-10
Y	AUTOIMMUNITY, Vol. 15, Suppl., issued 1993, H. L. Weiner, "Treatment of autoimmune diseases by oral tolerance to autoantigens," pages 6-7, see entire document.	1-13
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 174, issued October 1991, Miller et al., "Antigen-driven bystander Suppression after Oral Administration of Antigens," pages 791-798, see entire document.	1-13
Y	SCIENCE, Vol. 259, issued 26 February 1993, Weiner et al., "Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis," pages 1321-1324, see entire document.	1-10
Y	BRITISH JOURNAL OF RHEUMATOLOGY, Vol. 30, Suppl. 2, issued April 1991, N. A. Staines, "Oral tolerance and collagen arthritis," pages 40-43, see entire document.	1-11
Y	AUTOIMMUNITY, Vol. 16, issued 1993, Thompson et al., "Suppression of collagen induced arthritis by oral administration of type II collagen: Changes in immune and arthritic responses mediated by active peripheral suppression," pages 189-199, see entire document.	11
Y	SCIENCE, Vol. 261, issued 24 September 1993, Trentham et al., "Effects of oral administration of type II collagen on rheumatoid arthritis," pages 1727-1730, see entire document.	11

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Vol. 88, issued November 1991, Zhang et al., "Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin," pages 10252-10256, see entire document.	12
Y	JOURNAL OF EXPERIMENTAL MEDICINE, Vol. 178, issued July 1993, Rapoport et al., "Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice," pages 87-99, see entire document.	12
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 144, No. 5, issued 01 March 1990, Nussenblatt et al., "Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen," pages 1689-1695, see entire document.	13
Y	THE JOURNAL OF IMMUNOLOGY, Vol. 151, No. 10, issued 15 November 1993, Gregerson et al., "Oral tolerance in experimental autoimmune uveoretinitis," pages 5751-5761, see entire document.	13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/04120

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, drawn to methods of treating multiple sclerosis using bystander antigen and interferon.

Group II, claim 11, drawn to a second method, a method for treating for rheumatoid arthritis.

Group III, claim 12, drawn to a third method, a method for treating Type I diabetes.

Group IV, claim 13, drawn to a fourth method, a method for treating uveoretinitis.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the methods of Groups I-IV are directed to treatment of diseases which differ in their pathology and etiology and are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.